4. Preventive effects

4.1 Humans

4.1.1 Epidemiological studies

The proportion of total vitamin A intake that is contributed by preformed vitamin A (retinol and its esters) varies between populations, depending on a number of factors, including the balance of animal and plant foods and the prevalence of dietary supplements. In the United States, for example, preformed vitamin A makes up about 50% of the total dietary intake of vitamin A (Tee, 1992). Restricting this review to studies that separated the preformed vitamin A and carotenoid components would cause a number of potentially informative investigations to be omitted. Therefore, the Working Group chose to include studies that reported only total vitamin A (preformed and provitamin A) in the diet, but to present and discuss the findings separately from studies that specifically identified preformed vitamin A intake.

The review does not cover all studies that have reported on associations of vitamin A intake and cancer at any site. The Working Group chose to include cancer sites that have been reported in at least two studies and to exclude studies that were based on a small number of cases.

4.1.1.1 Methodological issues

(a) Case–control studies

Most of the studies relating vitamin A intake to cancer in humans are case–control studies. In these studies, individuals with cancer are compared with persons sampled from the general population with respect to prior consumption of vitamin A, or of foods rich in vitamin A. Advantages of such studies include efficiency and the capacity to study multiple exposures related to a single disease outcome. Often, studies of this kind provide the only information that is available. However, several methodological problems limit the ability of case–control studies to provide strong evidence for causal relations with vitamin A intake. Biased estimates of the relationship between vitamin A intake and disease may result if the controls are not representative of the population which gave rise to the cases (selection bias). For a case series at a hospital, for example, it may be difficult to define this underlying population, and even more difficult to sample it randomly, although in some instances hospital-based controls may give unbiased estimates of effect.

It may be difficult to obtain accurate information on past diet from cases and controls. In cancer studies, epidemiologists are usually interested in the diet of the cases months or years before the time the diagnosis is made. Estimates of diet at some time in the past are likely to be imprecise. Moreover, cases may differ systematically from controls in the accuracy of their description of their past diet (recall bias), particularly in view of the prominence given to diet–cancer hypotheses in some countries. For rapidly fatal cancers such as lung cancer, cases may be dead or too ill to participate by the time they are contacted, and information can be obtained only from a proxy

respondent, such as the spouse. This approach may yield unreliable data, particularly for a complex exposure such as dietary habits. These and other sources of error are important when studies are searching for small effects. In many studies of vitamin A, for instance, relative risks between the highest and lowest categories of intake are expected to be small and may be obscured by a relatively minor degree of bias.

The usual measure of effect in case–control studies is the odds ratio (OR; the relative odds of exposure among cases compared with the odds of exposure among controls). In a population-based case–control study, it can be shown that the odds ratio is an unbiased estimate of the measure of effect used in cohort studies (the relative risk (RR), i.e., the ratio of the incidence rate of the outcome among the exposed compared with the incidence rate in the unexposed). For simplicity, in this chapter, the term relative risk is used throughout.

(b) Cohort studies

In a cohort (prospective or follow-up) study, a group of persons whose dietary intake has been ascertained is followed over time with respect to disease incidence. Subsequent cases of cancer are compared with subjects who did not develop cancer, with respect to initial diet. The major advantage is that diet is assessed before the occurrence of disease, and thus should not be influenced by it. Selection bias should not occur, as the comparison group for the cases is explicit (the non-cases in the cohort). The major limitation of cohort studies is that a very large number of people (typically thousands or tens of thousands) must be enrolled and followed for many years to generate enough cancer diagnoses to achieve statistical power. For relatively uncommon cancer types or sites, a prospective study may never accrue enough cases. In addition, most cohort studies are limited to exposure data collected at the beginning of the study (unless blood or tissue samples are stored). Nonetheless, for vitamin A and the major cancers, prospective data are available, and generally constitute the best available sources of non-experimental evidence.

(c) Nested case–control studies

A common strategy in prospective studies using biomarkers of exposure is to employ a nested case–control design. It is usually prohibitively expensive and wasteful of samples to analyse all samples in a cohort. In a nested case–control study, typically most or all of the cases and a sample of non-cases (controls) are analysed. The study has the advantages of a prospective design, as the specimens providing the exposure data were collected before the diagnosis of cancer.

4.1.1.2 Assessment of exposure

The quality of the data on vitamin A and cancer in humans depends not only on study designs used, but also on the validity of the measurements of vitamin A intake. In general, intake can be measured by diet assessment (combining information on an individual's average intake of foods with the average vitamin A content of the foods), history of supplement use, or by measuring vitamin A (either retinol and/or retinyl esters) in the blood or other tissues.

(a) Diet assessment

Because certain foods (e.g., liver) are particularly rich sources of vitamin A, day-to-day variation in vitamin A intake is high. In many countries, certain foods such as milk are fortified with preformed vitamin A, and for some individuals, these foods account for a substantial proportion of their intake on some days. Thus, it is particularly important to assess intake of these foods over weeks or months, since one or a few days of intake (obtained for instance from 24-hour or three-day recalls) may be highly unrepresentative of long-term intake. Thus, many investigators use food frequency questionnaires, which generally seek to obtain information on average intake of foods over months or a year. The available data suggest that these instruments measure vitamin A intake reasonably well, although the effectiveness of questionnaires varies depending on the quality of the survey instrument, the circumstances in which information is sought and the skills and understanding of the study participants. For instance, the correlation between

total vitamin A intake estimated from a 61-item food frequency questionnaire compared with four weeks of diet records, in a population of women in the United States, was $r = 0.5$ ($p < 0.05$) (Willett *et al.*, 1985).

Many studies, particularly earlier ones, were not able to differentiate between preformed vitamin A (retinol and retinyl esters) and carotenoids with vitamin A activity, and thus reported data for total vitamin A, not preformed vitamin A specifically.

In contrast to some nutrients such as carotenoids (Ascherio *et al.*, 1992), validation of estimates of dietary intake of preformed vitamin A by comparison with serum levels is not feasible (see Section 4.1.1.2(*b*)).

A further issue is potential confounding of vitamin A by other nutrients in vitamin A-rich foods. For instance, dairy products are major sources of preformed vitamin A, but also have high levels of saturated fats. Thus, an artefactual positive association with preformed vitamin A may be observed at sites where cancer has a positive relation with saturated fat. Similarly, whenever total vitamin A intakes reflect intake of provitamin A-rich vegetables, confounding by other potentially anti-carcinogenic nutrients in these vegetables is a concern.

In the evaluation of dietary intake of retinol, it is worth distinguishing populations, chiefly North American ones, who have a relatively high probability of having been taking vitamin supplements, from other populations (e.g., southern Europe, Asia), where this practice is uncommon. When the use of vitamin supplements (mostly multivitamin preparations) has been specifically evaluated, this is mentioned in the description of the specific studies.

(b) Biochemical markers

Although serum retinol levels are depressed when vitamin A intake is sufficiently low to produce clinical deficiency, over the range of intake in generally well nourished populations serum retinol levels are only minimally related to vitamin A intake. For example, no material increase in blood retinol level was observed in a study in which 25 000 IU of preformed vitamin A (more than five times the usual dietary intake) was taken as a daily supplement for eight weeks (Willett *et al.*, 1983). Similarly, large supplements of β-carotene had no notable effect on blood retinol levels. Even among women with initially low values of plasma retinol, a daily supplement of 10 000 IU of vitamin A (fish-liver extract) increased blood levels only by 9%, although this was significant ($p < 0.02$) (Willett *et al.*, 1984a). Thus, serum retinol is not a valid indicator of vitamin A intake in vitamin A-sufficient subjects.

Case–control studies are particularly problematic for another reason: cancer and cancer treatment usually reduce retinol levels from pre-treatment values. Consequently, case–control studies that relied solely on blood retinol measures have been omitted from this review.

(c) Preformed vitamin A supplement use

In principle, users of preformed vitamin A supplements constitute a useful population to test the hypothesis that preformed vitamin A intake influences cancer risk. In practice, however, there are major limitations to this approach. In most populations, use of single vitamin A supplements is relatively rare; most preformed vitamin A supplements are taken in conjunction with other supplements, or in the form of multivitamins. This obviously limits the interpretation of information on preformed vitamin A supplement use. Vitamin supplements may also be used for relatively short periods, or in variable amounts, which further complicates interpretation of observational studies. Furthermore, supplement users tend to differ from non-supplement users in their exposure to established cancer risk factors (such as tobacco use) and socioeconomic status, suggesting that residual confounding due to these variables may remain even after statistical adjustment.

Patterson *et al.* (1997) reviewed vitamin supplement use and cancer risk in observational studies. Of eight prospective studies examining vitamin A supplement use and cancer at multiple sites, the only significant inverse association observed was for colon cancer among women, but not men, in a cohort in California, United States (Shibata *et al.*, 1992). Among case–control studies, the upper aerodigestive tract was the only site at which more than one study

reported a significant inverse association of cancer with vitamin A supplement use (see Table 10) (Patterson *et al.*, 1997). Patterson *et al.* (1997) concluded that further observational studies of supplement use 'appear warranted; however, methodological problems ... impair the ability to assess supplement use ... and cancer risk.' Because of the low prevalence of preformed vitamin A supplement use in most of the available studies, combined with the other methodological concerns, the Working Group did not systematically review the limited observational evidence relating supplement use to cancer risk.

4.1.1.3 Vitamin A and specific cancer sites

In the following review of the evidence relating vitamin A intake to specific cancers, studies are included which have presented data comparing relative risks for subjects according to categories of total and preformed vitamin A intake (high versus low). Details of these studies are presented in Tables 9–17. Studies that presented data only as a difference in mean vitamin A intake or blood retinol levels for cases and controls have been largely excluded, as were studies that reported only total cancer incidence as an endpoint.

(a) Lung cancer

The largest body of evidence on vitamin A and a specific cancer site exists for lung cancer (Table 9). Most of the case–control studies have reported an inverse association between total vitamin A intake and lung cancer, although some notable exceptions exist (Ziegler *et al.*, 1984; Jain *et al.*, 1990). In the study of Jain *et al.* (1990), increased consumption of vegetables was associated with significantly decreased risk, but the association was strongest for vegetables which are poor sources of vitamin A.

In general, among studies that have examined preformed vitamin A and carotenoid intake separately, no association or only a weak inverse association with preformed vitamin A is evident. In contrast, most of these studies have observed an inverse association with increased carotenoid consumption.

Prospective data are relatively sparse, with only four studies reporting more than 100 cases. Bjelke (1975) reported on 36 incident cases among 8278 Norwegian men who had earlier completed a mailed dietary questionnaire. Kvåle *et al.* (1983), in the further follow-up of the extended cohort (168 cases), observed a relative risk of 0.5 for high versus low intake of total vitamin A. This inverse association was mostly attributable to intake of carrots and other vegetables, with some additional contribution from milk. Thus, this study is more consistent with a beneficial effect of carotenoid sources of vitamin A than one of preformed vitamin A.

Shekelle *et al.* (1981) reported data on the independent associations of carotenoids and preformed vitamin A. In 19 years of follow-up of 2107 men, 33 cases occurred. Preformed vitamin A intake was weakly positively associated with disease risk, but a strong inverse association was observed for carotenoid intake, which was similar in magnitude for smokers and non-smokers. Paganini-Hill and colleagues (1987) observed little protective effect of total vitamin A or preformed vitamin A, but a modest decrease in lung cancer risk among both men and women in the upper tertile of carotenoid intake. Knekt *et al.* (1991a), in a follow-up of Finnish men, observed a weak positive relationship with preformed vitamin A among smokers. Yong *et al.* (1997) observed little evidence of any association with total vitamin A (based on 24-h recall estimates of dietary intake), and a relative risk of 1.3 for high versus low intake of preformed vitamin A.

As discussed earlier, blood retinol levels are poor indicators of preformed vitamin A intake. However, blood carotenoids do reflect carotenoid intake. A protective effect of being in the highest category of blood β-carotene level or total carotenoid intake has been a remarkably consistent finding in the nested case–control studies. In none of the nested case–control studies was a significant association with blood retinol levels observed.

In summary, the observational studies, including both case–control and prospective evidence, support a protective effect of higher intakes of foods containing carotenoids on risk of lung cancer. The possibility that other components of carotenoid-rich foods may be

Table 9. Studies of vitamin A intake and lung cancer

Reference, site	No. of cases[a]	Categories[b]	Total vitamin A[c]		Preformed vitamin C[c]		Vit.	Proxies	Adjusted for
			RR	95% CI	RR	95% CI	supp.[d]	(% cases)	
Case–control studies									
Mettlin *et al.* (1979), USA	292 M	Three	[0.6]	$p < 0.05$	–	–	N	0	Age, smoking
Gregor *et al.* (1980), UK	78 M	Three	[0.5]	p, trend < 0.05	–	–	Y	0	Unadjusted
	22 F		[1.9]	p, trend < 0.05					
Hinds *et al.* (1984), USA	364	Quartiles	[0.7]	[0.5–1.1]	–	–	Y	24%	Age, ethnicity, sex, smoking, cholesterol intake, occupation
Ziegler *et al.* (1984), USA	763 M, white	Quartiles	[1.1][f]	p, trend = 0.30	[1.3]	p, trend = 0.11	Y	60%[e]	Age, race, residence, smoking
Byers *et al.* (1984), USA	427 M	Tertiles	1.2[g]	p, trend = 0.73	–	–	N	0	Age, smoking
			0.7[d]	p, trend = 0.07					
			0.6[h]	p, trend = 0.09					
Samet *et al.* (1985), USA	332 Anglo	Tertiles	[0.6]	[0.4–1.0]	[1.1]	[0.7–1.7]	Y	47%	Age, ethnicity, sex, smoking
	125 Hispanic		[1.1]	[0.6–2.0]	[1.7]	[0.8–3.3]			
Wu *et al.* (1985), USA	220 F	Quartiles	–	–	[0.8]	[0.4–2.0]	Y	0	Age, residence, smoking
		Dichotomous			[1.0]	[0.4–2.5]			
Byers *et al.* (1987), USA	296 M	Quartiles	[0.7]	p, trend = 0.12	–		N	0	Age, smoking
	154 F		[0.8]	p, trend = 0.4	–				
Pastorino *et al.* (1987), Italy	47 F	Tertiles	–	–	[0.3]	[0.1-1.2]	N	0	Age, smoking, serum cholesterol, triglycerides
Fontham *et al.* (1988), USA	1253	Tertiles	–	–	0.9	0.7–1.1	N	27%	Age, race, sex, smoking
Koo (1988), Hong Kong	88 F, Chinese non-smokers	Three	–	–	[0.4]	p, trend = 0.02	N	0	Age, parity, education
Ho *et al.* (1988), Singapore	50 M, Chinese	Quartiles	[0.6]	[0.2–2.0]	–	–	Y	0	Age, ethnicity, sex
Le Marchand *et al.* (1989), USA	230 M	Quartiles	[0.6]	p, trend = 0.003	[1.1]	p, trend = 0.70	Y	29%	Age, ethnicity, smoking
	102 F		[0.4]	p, trend = 0.14	[1.0]	p, trend = 0.75			
Dartigues *et al.* (1990), France	106	< 3 330 IU	–	–	[0.2]	[0.1–0.4]	N	0	Age, residence, sex, smoking, alcohol, occupation
Jain *et al.* (1990), Canada	839	Quartiles	1.2	p, trend = 0.46	1.2	p, trend = 0.54	Y	34%	Age, residence, sex, smoking
Kalandidi *et al.* (1990), Greece	91 F, non-smokers	Quartiles	–	–	1.3	0.98–1.8	N	0	Age, education, energy
Candelora *et al.* (1992), USA	124 F non-smokers	Quartiles	–	–	1.2	0.6–2.4	Y	0	Age, education, energy

Table 9. (contd)

Reference, site		No. of cases[a]	Categories[b]	Total vitamin A[c]		Preformed vitamin A[c]		Vit.	Proxies	Adjusted for
				RR	95% CI	RR	95% CI	supp.[d]	(% cases)	
Cohort studies	Cohort size									
Kvåle *et al.* (1983), Norway	16 713 M	153	Four	[0.6]	*p*, trend < 0.04	–	–	N	0	Unadjusted
Paganini-Hill *et al.* (1987), USA	10 473	37 M	Tertiles	[0.9]	*p*, trend > 0.05	[0.8]	*p*, trend > 0.05	Y	0	Age
		18 F		[0.9]	*p*, trend > 0.05	[0.7]	*p*, trend > 0.05			
Knekt *et al.* (1991a), Finland	4538 M		Tertiles					Y	0	Age, smoking
	non-smokers	18		–	–	[0.7]	*p*, trend = 0.72			
	smokers	99		–	–	[1.4]	*p*, trend = 0.08			
Yong *et al.* (1997), USA	10 068	248	Quartiles	1.0	0.7–1.5	1.3	0.9–1.8	Y	0	Age, race, education, activity, body mass index, family history, energy, smoking, alcohol

Reference, site	No. of cases	No. of controls	Categories	Blood retinol[c]		Adjusted for
				RR	95% CI	
Prospective blood-based studies						
Friedman *et al.* (1986), USA	151	302	Quintiles	0.8	*p*, trend = 0.94	Age, sex, skin colour, smoking
Menkes *et al.* (1986), USA	99	196	Quintiles	1.1	*p*, trend = 0.68	Age, sex, race, smoking
Knekt *et al.* (1990), Finland	108 M	204	Quintiles	1.5	0.3–1.3	Age, sex, residence, smoking
	6 F	12		1.4	0.2–10.0	
Eichholzer *et al.* (1996), Switzerland	87 M	2173	Octiles			Age, smoking, lipids
	> 60 y			[0.4]	[0.8–8.1]	
	≤ 60 y			[0.7]	[0.3–1.7]	

[a] Study populations include both men and women unless otherwise stated

[b] Number of categories used (if quantiles not used); otherwise number of quantiles used for categorization

[c] Relative risk for highest versus lowest category

[d] Vitamin supplement use assessed (Y) or vitamin supplement use not assessed, or unclear if it was assessed (N)

[e] For cases and controls combined

[f] Adenocarcinoma

[g] Squamous-cell carcinoma

[h] Small cell carcinoma

responsible for an apparent effect of carotenoids deserves further exploration. The data support the conclusion that dietary intake of preformed vitamin A does not influence lung cancer risk.

(b) Upper aerodigestive tract cancer

Five case–control studies (four from the United States and one from Australia) (Table 10) evaluated the relationship between dietary intake of total vitamin A or preformed vitamin A and the risk of cancer of the oral cavity and pharynx. Two studies (McLaughlin *et al.*, 1988; Gridley *et al.*, 1990) found direct associations which were significant in males. These included the largest investigation on the topic (831 cases and 979 controls) which yielded RRs of 1.6 in men (p, trend = 0.007) and 1.4 (p, trend = 0.27) in females in the highest quartile of intake (McLaughlin *et al.*, 1988). In one study (Marshall *et al.*, 1982), a significantly reduced risk was reported for the highest tertile for vitamin A intake. The relationship between cancer of the oesophagus and retinol intake was evaluated in eight case–control studies. Approximately two-fold elevated RRs were reported in four investigations, including the largest from Calvados, France (743 cases and 1975 controls) (Tuyns *et al.*, 1987) and another from the United States, where adjustment for total energy intake was possible (Graham *et al.*, 1990a). No study suggested risk reduction in individuals who reported high retinol intake. For cancer of the larynx, three case–control investigations suggested either an elevated risk after allowance for total energy intake (Freudenheim *et al.*, 1992) or no effect on risk modification (Mackerras *et al.*, 1988; Estève *et al.*, 1996). The latter was a large collaborative European study (1147 cases and 3057 controls), where cancer of the hypopharynx was also evaluated and an RR of 0.6 was observed in the highest quartile for preformed vitamin A intake.

A prospective study of postmenopausal women in Iowa, United States, reported an RR of 0.9, (95% CI, 0.4–2.2) based on 33 cases of cancer of the oral cavity, pharynx and oesophagus (Zheng *et al.*, 1995).

Nomura *et al.* (1997a) evaluated the association between serum levels of various micronutrients and cancer incidence in a cohort of 6832 American men of Japanese ancestry. Serum levels were measured on average six years before the diagnosis of cancer of the oral cavity and pharynx (16 cases), oesophagus (28) or larynx (23). Mean levels of retinol and total retinoids were very similar in cancer cases and 138 controls.

Among the studies listed in Table 10, four reported RRs for intake of vitamin A supplements and/or multivitamin preparations. These tended to be systematically below unity. Gridley *et al.* (1992), in an expanded re-evaluation of the study by McLaughlin *et al.* (1988) on 1114 cases of cancer of the oral cavity and pharynx and 1268 controls, reported an RR of 0.4 (95% CI, 0.2–0.8) for 10 or more years of vitamin A supplement use. This was seen consistently in men and women but after adjustment for vitamin E intake (the strongest protective factor) became 0.6 (95% CI, 0.3–1.4).

(c) Gastric cancer

Almost all the published epidemiological data on the relationship between vitamin A and gastric cancer are from case–control studies (Table 11). Stehr *et al.* (1985) observed a positive association between total vitamin A intake, as reported by next-of-kin of deceased gastric cancer cases, and gastric cancer risk; the use of proxy interviews increases the likelihood of misclassification and bias in this study. Risch *et al.* (1985) observed a strong inverse association with β-carotene but not preformed vitamin A; inverse associations with β-carotene but not retinol have been reported by La Vecchia *et al.* (1987a), You *et al.* (1988), Buiatti *et al.* (1990), Gonzalez *et al.* (1994) and Hansson *et al.* (1994). A strong positive association with preformed vitamin A intake was reported by Graham *et al.* (1990b), along with a significant inverse association with β-carotene. In a study in Milan, Italy by La Vecchia *et al.* (1994), preformed vitamin A intake was not associated with cancer risk.

In a prospective study, Nomura *et al.* (1995) observed essentially no relationship between serum retinol levels and subsequent risk of gastric cancer, but a modest inverse trend with β-carotene levels.

Table 10. Total vitamin A and retinol intake and risk of upper aerodigestive cancers

Reference, site	Cancer site	No. of cases[a]	Categories[b]	Total vitamin A[c]		Preformed vitamin A[c]		Vit. supp.[d]	Proxies (% cases)	Adjusted for
				RR	95% CI	RR	95% CI			
Case-control studies										
Marshall *et al.* (1982), USA	Oral cavity	425	Tertiles	0.5	*p*, trend < 0.05	–	–	N	0	Age, alcohol, smoking
McLaughlin *et al.* (1988), USA	Oral cavity and pharynx	871	Quartiles	–	–	M 1.6 F 1.4	*p*, trend = 0.007 *p*, trend = 0.3	Y	0	Smoking, alcohol
Rossing *et al.* (1989), USA	Pharynx	166	Quartiles	–	–	1.1	0.6–2.0	Y	52%	Age, smoking, alcohol
Gridley *et al.* (1990), USA	Oral cavity and pharynx	190	Quartiles	–	–	M 4.5 F 0.6	*p*, trend = 0.001 *p*, trend = 0.8	N	29%	Alcohol, smoking, energy
Kune *et al.* (1993), Australia	Oral cavity and pharynx	41	Tertiles	–	–	0.6	0.3–1.4	N	0	Age
Ziegler *et al.* (1981), USA	Oesophagus	120	Tertiles	0.7	*p*, trend > 0.05	–	–	N	100%	Alcohol
Tuyns *et al.* (1987), France	Oesophagus	743	4 levels	1.0	0.7– 1.6	2.1	1.5–3.0	N	0	Alcohol, smoking
Brown *et al.* (1988), USA	Oesophagus	207 M	Tertile	–	–	1.9	1.0–3.5	N	0 (diet)	Smoking, alcohol
Decarli *et al.* (1987), Italy	Oesophagus	105	Three	–	–	2.3	1.1–4.5	Y	0	Age, education, social class, body mass index, alcohol, smoking
Graham *et al.* (1990a), USA	Oesophagus	176	Quartiles	2.1	1.1–4.0	3.0	1.6–5.8	N	0	Age, education, alcohol, tobacco, energy

Table 10. (contd)

Reference, site	Cancer site	No. of cases[a]	Categories[b]	Total vitamin A[c]		Preformed vitamin A[c]		Vit. supp.[d]	Proxies (% cases)	Adjusted for
				RR	95% CI	RR	95% CI			
Gao *et al.* (1994), China	Oesophagus	902	Quartiles	–	–	M 0.9 F 1.3	*p*, trend = 0.8 *p*, trend = 0.1	N	0	Age, education, birth place, tea, smoking, alcohol, energy
Hu *et al.* (1994), China	Oesophagus	196	Quartiles	–	–	1.3	0.7–2.4	Y		Alcohol, smoking, occupation
Tavani *et al.* (1994), Italy	Oesophagus	46	Tertiles	–	–	1.1	0.5–2.7	Y	0	Education, age, smoking
Mackerras *et al.* (1988), USA	Larynx	151	Tertiles	[0.8]	[0.4–1.4]	[1.1]	[0.6–2.5]	N	0	Age, smoking, alcohol
Freudenheim *et al.* (1992), USA	Larynx	250 M	Quartiles	0.6	0.3–1.04	2.8	1.4–5.6	N	0	Education, alcohol, smoking, total energy
Estève *et al.* (1996), Europe, six regions	Larynx Hypopharynx + epilarynx	727 M 399 M	Five	–	–	1.0 0.6	0.7–1.4 0.4–0.9	N	0	Age, alcohol, smoking, centre
Cohort studies	Cancer site	Cohort size								
Zheng *et al.* (1995), USA	Mouth, pharynx, oesophagus	34691 33	Tertiles	–	–	0.9	0.4–2.2	Y	0	Age, smoking, energy

[a] Study populations include both men and women unless otherwise stated

[b] Number of categories used (if quantiles not used); otherwise number of quantiles used for categorization

[c] Relative risk for highest versus lowest category

[d] Vitamin supplement use assessed (Y) or vitamin supplement use not assessed, or unclear if it was assessed (N)

Table 11. Studies of vitamin A intake and gastric cancer

Reference, site	No. of cases[a]	Categories[b]	Total vitamin A[c]		Preformed vitamin A		Vit. supp.[d]	Proxies	Adjusted for
			RR	95% CI	RR	95% CI		(% cases)	
Case–control studies									
Stehr *et al.* (1985), USA	111	Two	1.7	1.02–3.1	–	–	N	100	Year of death, age, sex, race, residence
Risch *et al.* (1985), Canada	246	Per 10 000 IU	–	–	0.9	0.4–1.8	N	0	Sex, age, residence
La Vecchia *et al.* (1987a), Italy	206	Tertiles	–	–	0.8	*p*, trend = 0.4	N	0	Sex, age, residence, education
You *et al.* (1988), China	564	Quartiles	–	–	1.0	0.7–1.4	N	0	Sex, age, income
Graham *et al.* (1990b), USA	186 M	Four	–	–	3.1	1.7–5.7	N	0	Sex, age, residence
	107 F	Three			2.1	1.1–4.1			
Buiatti *et al.* (1990), Italy	1016	Quintiles	–	–	1.0	0.7–1.3	Y	0	Sex, age
Hansson *et al.* (1994), Sweden	338	Quartiles	0.8	0.5 – 1.1	0.8	0.6–1.3	N	0	Sex, age, energy intake
Gonzalez *et al.* (1994), Spain	354	Quartiles	–	–	1.5	*p*, trend = 0.1	N	0	Sex, age, residence, energy intake
La Vecchia *et al.* (1994), Italy	723	Quintiles	–	–	0.9	0.7–1.3	N	0	Age, sex, education, family history, body mass index, energy

Prospective blood-based studies	No. of cases	No. of controls		Blood retinol				
				RR	95% CI			
Nomura *et al.* (1995), USA	70	302	Three	–	–	1.2	0.6–2.2	Age

[a] Study populations include both men and women unless otherwise stated

[b] Number of categories used (if quantiles not used); otherwise number of quantiles used for categorization

[c] Relative risk for highest versus lowest category

[d] Vitamin supplement use assessed (Y) or vitamin supplement use not assessed, or unclear if it was assessed (N)

Thus, a substantial body of data suggests that components of carotenoid-rich vegetables are protective against gastric cancer, but no data suggest that preformed vitamin A has such an influence.

(d) Colon cancer

Although many studies have examined fat and fibre intake in relation to colon cancer risk, relatively few have reported data on vitamin A. McKeown-Eyssen and Bright-See (1984) reported from an international correlation study a weak positive relationship between preformed vitamin A intake and colon cancer mortality (r = 0.27), after adjustment for animal fat and cereal fibre intake; there was essentially no association with intake of vegetables and fruits (the main sources of carotenoids).

In a case–control study in Australia, Potter and McMichael (1986) did not observe a substantial protective association of preformed vitamin A or carotenoids among either men or women (Table 12). Lyon *et al.* (1987), working in Utah, United States, observed no overall relationship, although in sex-specific analyses, a modest protective association with total vitamin A intake among women was observed (adjusted for age and energy only). In a subsequent case–control study in Utah, West *et al.* (1989) observed a significant inverse association with higher intake of β-carotene after adjusting for age, obesity, crude fibre and energy intake; no significant association was observed for total vitamin A. Graham *et al.* (1988), however, found no protective association in a study performed in New York State, United States.

In a case–control study (Ferraroni *et al.*, 1994) carried out in Milan, Italy, between 1985 and 1992, high retinol intake was inversely associated with colon cancer (RR in highest versus lowest intake quintile, 0.7; 95% CI, 0.5–0.9) but not rectal cancer risk (corresponding RR, 0.8; 95% CI, 0.6–1.1).

Another large case–control study on cancer of the colon and rectum was carried out between 1992 and 1996 in six areas of Italy, using a more detailed, validated food frequency questionnaire and food composition tables (La Vecchia *et al.*, 1997). Intake level of retinol was not associated with either colon or rectal cancer.

Few prospective studies have reported data on colon cancer. Paganini-Hill *et al.* (1987) did not observe any substantial protective effect of either preformed vitamin A or provitamin A carotenoids. Heilbrun *et al.* (1989) observed a modest non-significant inverse association between higher intake of total vitamin A and both colon cancer ([RR, 0.7]; p, trend = 0.1) and rectal cancer ([RR, 0.8]; p, trend = 0.4). A similar inverse association was observed between preformed vitamin A intake for colon ([RR=0.7]; p, trend = 0.2) but not rectal (RR, 1.0; p, trend = 0.8) cancer. Interestingly, in a nested case–control study in Maryland, United States, subjects in the upper quintile of serum retinol were at reduced risk of colon cancer [RR, 0.3; 95% CI, 0.1–0.8] after up to nine years of follow-up, although this observation should not be given much weight given the limitations of serum retinol levels (Schober *et al.*, 1987). Nomura *et al.* (1985) observed higher median levels of retinol, but somewhat lower levels of β-carotene among men who subsequently developed colon cancer, compared with controls.

In summary, there is little evidence to suggest that vitamin A is protective against colon cancer. The data are sparse and inconsistent, however. As animal fat and fibre may be important determinants of colon cancer, more studies which carefully control for these are needed.

(e) Skin cancer

A few studies have investigated the relationship of vitamin A to skin cancer. Table 13 lists those concerned with non-melanocytic cancers. A mix of prospective and case–control (Middleton *et al.*, 1986; Kune *et al.*, 1992; Hunter *et al.*, 1992) studies conducted among Caucasian populations with a wide range of disease risk reported no effect of vitamin A intake: although the risk estimates in the individual studies were generally greater than unity, in every instance, the 95% confidence interval included 1.0. In addition, two prospective studies have reported on the relationship between pre-diagnostic levels of

Table 12. Studies of vitamin A intake and colon cancer

Reference, site	No. of cases[a]	Categories[b]	Total vitamin A[c]		Preformed vitamin[c]		Vit. supp.[d]	Proxies (% cases)	Adjusted for
			RR	95%	RR	95% CI			
Case–control studies									
Potter & McMichael (1986), Australia	121 M colon 99 F colon 124 M rectum 75 F rectum	Quintiles	–	–	1.4 1.6 0.7 1.3	0.8–2.4 0.7–3.5 0.4–1.4 0.6–3.0	N	0	Age
Lyon *et al.* (1987), USA	246	Four	M 1.4 F 0.7	– –	–	–	N	0	Age, county, race, energy
West *et al.* (1989), USA	112 M 119 F	Four	0.7 1.5	0.3–1.8 0.6–3.9	–	–	N	0	Age, body mass index, crude fibre, energy
Ferraroni *et al.* (1994), Italy	828 colon 498 rectum	Quintiles	–	–	0.7 0.8	0.5–0.9 0.6–1.1	N	0	Age, sex, education, family history, body mass index, energy
La Vecchia *et al.* (1997), Italy	1225 colon 728 rectum	Quintiles	–	–	1.1 1.0	1.0–1.2 0.9–1.1	N	0	Age, residence, sex, education, activity, energy, fibre
Cohort studies	Cohort size	No. of cases							
Paganini-Hill *et al.* (1987), USA	6694	52 M 58 F	Tertile	[1.3] [0.8]	*p*, trend > 0.05 *p*, trend > 0.05	[1.3] [1.0]	*p* , trend > 0.05 *p* , trend > 0.05	Y	0	Age
Heilbrun *et al.* (1989), USA	8006 M	102 colon 60 rectum	Quintiles	[0.7] [0.8]	*p*, trend = 0.1 *p*, trend = 0.4	[0.7] [1.0]	*p*, trend = 0.2 *p*, trend = 0.8	N	0	Age Age and alcohol
Prospective blood-based studies	No. of cases	No. of controls			Blood retinol[c]				
					RR	95% CI			
Schober *et al.* (1987), USA, Maryland	72	143	Quintiles	–	–	0.3	0.1–0.8			Age, sex, residence

[a] Study populations include both men and women unless otherwise stated

[b] Number of categories used (if quantiles not used); otherwise number of quantiles used for categorization

[c] Relative risk for highest versus lowest category

[d] Vitamin supplement use assessed (Y) or vitamin supplement use not assessed, or unclear if it was assessed (N)

Table 13. Studies of vitamin A intake and non-melanocytic skin cancers

Reference, site	No. of cases[a]		Categories[b]	Total vitamin A[c]		Preformed vitamin A[c]		Vit. supp.[d]	Proxies (% cases)	Adjusted for
				RR	95% CI	RR	95% CI			
Case–control studies										
Middleton *et al.* (1986), USA [SCC + BCC]	606 M 161 F		Tertiles	1.13 0.94	*p*, trend = 0.40	–	–		0	Age, smoking, alcohol
Cohort studies	Cohort size	No. of cases								
Knekt *et al.* (1990), Finland [BCC]	36 265	38 M 29 F	Quartiles	–	–	1.7	0.5–5.1		0	Smoking
Hunter *et al.* (1992), USA [BCC]	73 366 F	771		–	–	1.0	0.8 –1.2	Y	0	Age, residence, body mass index, hair colour, skin type, history of sunburn
Karagas *et al.* (1997), USA [SCC]	1805	132	Quartiles	–	–	1.4	0.8–2.6		0	Age, smoking, sex, study centre
Prospective blood-based studies	No. of cases	No. of controls				Blood retinol[c]				
						RR	95% CI			
Breslow *et al.* (1995), USA BCC	32	32	Tertiles	–	–	3.3	0.9–11.6			Age, sex, race
SCC	37	37				1.8	0.6–5.8			

[a] Study populations include both men and women unless otherwise stated

[b] Number of categories used (if quantiles not used); otherwise number of quantiles used for categorization

[c] Relative risk for highest versus lowest category

[d] Vitamin supplement use assessed (Y) or vitamin supplement use not assessed, or unclear if it was assessed (N)

SCC = squamous cell carcinoma, BCC = basal cell carcinoma

retinol and melanoma (Breslow *et al.*, 1995; Knekt *et al.*, 1991b), and both reported no significant association on the basis of 30 and 10 cases, respectively. This is consistent with the findings of a case–control study of melanoma and dietary intake of preformed vitamin A (Kirkpatrick *et al.*, 1994).

(f) Breast cancer

A relatively large number of studies have assessed the association between vitamin A and breast cancer (Table 14). Of the six case–control studies which have reported data for total vitamin A, all reported an inverse association between intake and risk. Four of the case–control studies that reported on the association with preformed vitamin A observed modest decreases in risk with higher intake (Katsouyanni *et al.*, 1988; Marubini *et al.*, 1988; Zaridze *et al.*, 1991; Longnecker *et al.*, 1997), while another seven studies found essentially no association (La Vecchia *et al.*, 1987b; Rohan *et al.*, 1988; Toniolo *et al.*, 1989; Ingram *et al.*, 1991; Richardson *et al.*, 1991; Yuan *et al.*, 1995; Negri *et al.*, 1996). In a meta-analysis of 12 case–control studies by Howe *et al.* (1990), the relative risk in the highest quintile for total vitamin A intake was 0.9 ($p = 0.04$), for β-carotene 0.9 ($p = 0.007$) and for preformed vitamin A 1.0 ($p = 0.52$).

In summary, the case–control data for breast cancer are compatible with a modest inverse association with higher intakes of vitamin A, and, as for lung cancer, this association is somewhat stronger for total vitamin A than for preformed vitamin A.

In a follow-up of female residents of a Californian retirement community, 123 breast cancers occurred (Paganini-Hill *et al.*, 1987). The relative risk comparing the highest tertile of total vitamin A intake with the lowest tertile was [0.8] ($p > 0.05$); for preformed vitamin A this risk was [0.7] ($p > 0.05$). In a follow-up of 89 494 nurses in the United States, Hunter *et al.* (1993) reported a relative risk of 0.8 (95% CI, 0.7–1.0) for women in the highest quintile of total vitamin A intake compared with the lowest. The comparable relative risk for preformed vitamin A was 0.8 (95% CI 0.7–1.0). The association for total vitamin A was slightly stronger among premenopausal (RR = 0.8) than postmenopausal (RR = 0.9) women. Rohan *et al.* (1993) observed suggestive evidence of an inverse association for both total vitamin A and preformed vitamin A. However, the studies of Graham *et al.* (1992), Kushi *et al.* (1996a) and Verhoeven *et al.* (1997), all conducted among postmenopausal women, observed essentially no relationship with disease risk for either total vitamin A or preformed vitamin A.

Studies of blood retinol and breast cancer risk are very limited. Knekt *et al.* (1990) reported a non-significantly lower risk associated with higher retinol levels at baseline.

(g) Prostate cancer

Graham *et al.* (1983), in a case–control study in New York State, observed a positive association between higher intakes of total vitamin A and risk of prostate cancer. In a case–control study among black men in Washington, DC, United States, Heshmat *et al.* (1985) also observed a positive association, which was statistically significant in the subgroup of men aged 30–49 years. Kolonel *et al.* (1988) reported a significantly elevated relative risk for consumption of high levels of total vitamin A but not preformed vitamin A among men 70 years or older in Hawaii, United States (the findings were essentially null for men aged < 70 years).

Most subsequent studies have not confirmed this increased risk (Table 15). In a reanalysis of the case–control study in Hawaii, the excess risk was almost entirely attributable to increased consumption of papaya (a food very high in carotenoids, not retinol) among cases. No elevation in risk was observed for β-carotene from other food sources (Le Marchand *et al.*, 1991). In Japan, Ohno *et al.* (1988) observed an inverse association for β-carotene, as did Mettlin *et al.* (1989) in New York State, Ross *et al.* (1987) in California African-Americans (the relation was null among whites) and Rohan *et al.* (1995) among Canadian men. West *et al.* (1991) observed an elevation in risk with higher intake of vitamin A for men 68 years of age or older, but not among younger men. Talamini *et al.* (1992) observed

Table 14. Studies of vitamin A intake and breast cancer

Reference, site	No. of cases[a]	Categories[b]	Total vitamin A[c]		Preformed vitamin A[c]		Vit. supp.[d]	Proxies (% cases)	Adjusted for
			RR	95% CI	RR	95% CI			
Case-control studies									
Graham *et al.* (1982), USA	2024	Four	[0.8]	*p*, trend < 0.05	–	–	N	0	Age
La Vecchia *et al.* (1987b), Italy	1108	Three	–	–	0.9	0.7–1.1	N	0	Age, education, family history, body mass index, reproductive factors
Katsouyanni *et al.* (1988), Greece	120	Quintiles	0.5	0.3–0.8	0.6	0.4–1.0	N	0	Age, education, residence, reproductive factors
Marubini *et al.* (1988), Italy	214	Quintiles	–	–	0.7	0.4–1.5	N	0	Age, energy, alcohol
Rohan *et al.* (1988), Australia	451	Quintiles	–	–	1.2	0.8–1.8	N	0	Age
Toniolo *et al.* (1989), Italy	250	Quartiles	–	–	1.2	*p*, trend > 0.05	N	0	Age, energy
Potischman *et al.* (1990), USA	83	Quartiles	[0.7]	[0.3–2.0]	–	–	Y	0	Age, family history, income, body mass index, reproductive factors
Ingram *et al.* (1991), Australia	99	Quartiles	–	–	1.0	0.6–1.7	N	0	Age, residence
Zaridze *et al.* (1991), USSR	81	Quartiles	0.2	0.0–0.8	0.5	0.1–1.7	N	0	Age, energy, education, reproductive factors
Richardson *et al.* (1991), France	409	Tertiles	–	–	1.5	1.0– 2.1	N	0	Age, education, family history, benign breast disease, reproductive factors
London *et al.* (1992), USA	313	Quintiles	–	–	0.7	0.4–1.3	Y	0	Age, energy, alcohol, body mass index, family history, reproductive factors
Holmberg *et al.* (1994), Sweden	265	Quartiles	–	–	0.7	0.4–1.0	N	0	Age, residence, energy
Yuan *et al.* (1995), China	834	per 1753 IU	–	–	0.9	0.6–1.2	N	0	Age, family history, reproductive factors
Negri *et al.* (1996), Italy	2569	Quintiles	0.7	0.6–0.9	0.9	0.7–1.0	N	0	Age, centre, education, energy, alcohol, reproductive factors
Longnecker *et al.* (1997), USA	3543	Six	0.4	0.1–1.1	0.6	0.3–1.2	Y	0	Age, centre, body mass index, education, benign breast disease, family history, alcohol, reproductive factors

Table 14. (contd)

Reference, site	Cohort size	No. of cases	Categories[b]	Total vitamin A[c]		Preformed vitamin A[c]		Vit. supp.[d]	Proxies (% cases)	Adjusted for
				RR	95% CI	RR	95% CI			
Cohort studies										
Paganini-Hill *et al.* (1987), USA	[~4000]	123	Tertiles	[0.8]	*p*, trend > 0.05	[0.7]	*p*, trend > 0.05	Y	0	Age
Graham *et al.* (1992), USA	18 586	344	Quintiles	1.0	0.7–1.3	0.9	0.7–1.3	Y	0	Age, education
Hunter *et al.* (1993), USA	89 494	1439	Quintiles	0.8	0.7–1.0	0.8	0.7–1.0	Y	0	Age, energy, fat intake, family history, body mass index, alcohol, benign breast disease, reproductive factors
Rohan *et al.* (1993), Canada	56 837	519	Quintiles	0.8	0.6–1.2	0.8	0.6–1.2	Y	0	Age, energy, education, family history, benign breast disease, reproductive factors
Kushi *et al.* (1996a), USA	34 387	879	Quintiles	0.9	0.7–1.1	1.0	0.8–1.2	Y	0	Age, energy, education, family history, benign breast disease, body mass index, reproductive factors
Verhoeven *et al.* (1997), Netherlands	62 573	650	Quintiles	–	–	1.2	0.8–1.8	Y	0	Age, energy, family history, benign breast disease, alcohol, reproductive factors

Prospective blood-based studies	No. of cases	No. of controls		Blood retinol[c]		Adjusted for
				RR	95% CI	
Wald *et al.* (1984), Guernsey	78	39	Quintiles	0.8	*p*, trend > 0.05	Age
Knekt *et al.* (1990), Finland	93	52	Quintiles	[0.8]	[0.4–2.0]	Age, residence, smoking

[a] Study populations include both men and women unless otherwise stated

[b] Number of categories used (if quantiles not used); otherwise number of quantiles used for categorization

[c] Relative risk for highest versus lowest category

[d] Vitamin supplement use assessed (Y) or vitamin supplement use not assessed, or unclear if it was assessed (N)

Table 15. Studies of vitamin A intake and prostate cancer

Reference, site	No. of cases[a]	Categories[b]	Total vitamin A[c]		Preformed vitamin A[c]		Vit. supp.[d]	Proxies (% cases)	Adjusted for
			RR	95% CI	RR	95% CI			
Case–control studies									
Graham *et al.* (1983), USA	262	Quartiles	1.8	*p*, trend < 0.01	–	–	N	0	Age
Ross *et al.* (1987), USA	142 Blacks 142 Whites	Tertiles	0.8 1.0	*p* > 0.05 *p* > 0.05	– –	– –	–	0	Age, residence, race
Ohno *et al.* (1988), Japan	100	Quartiles	[0.4]	[0.2–0.8]	–	–	N	0	Age
Kolonel *et al.* (1988), USA	189 (< 70 y) 263 (> 70 y)	Quartiles	0.8 2.0	*p*, trend = 0.16 *p*, trend <0.01	0.9 1.4	*p*, trend = 0.82 *p*, trend = 0.10	Y	0	Age, ethnicity
West *et al.* (1991), USA	179 (<68 y) 179 (≥68 y)	Quartiles	1.0 1.6	0.6–1.7 0.9–2.7	– –	– –	Y	0	Age, residence
Rohan *et al.* (1995), Canada	207	Quartiles	0.8	*p*, trend = 0.1	0.6	*p*, trend = 0.02	Y	0	Age, energy
Ghadirian *et al.* (1996), Canada	232	Quartiles	0.9	*p*, trend = 0.87	0.8	*p*, trend = 0.15	N	0	Age, energy, family history
Talamini *et al.* (1992), Italy	157 (< 70 y) 114 (≥ 70 y)	Tertiles	–	–	0.8 2.2	0.5–1.3 1.1–4.2	N	0	Age, residence, education, body mass index
Andersson *et al.* (1996), Sweden	526	Quartiles	1.3	*p*, trend = 0.43	1.3	*p*, trend = 0.10	N	0	Age, energy

Table 15 (contd)

Reference, site	Cohort size	No. of cases [a]	Categories[b]	Total vitamin A[c]		Preformed vitamin A		Vit. supp. [d]	Proxies (% cases)	Adjusted for
				RR	95% CI	RR	95% CI			
Cohort studies										
Paganini-Hill *et al.* (1987), USA		93	Tertiles	1.4	$p > 0.05$	[1.1]	p, trend > 0.05	Y	0	Age
Hsing *et al.* (1990a), USA	17 633	78 (< 75 y) 71 (>75 y)	Quartiles	2.8 0.4	p, trend < 0.05 p, trend = 0.01	1.7 0.9	p, trend > 0.05 p, trend = 0.05	N	0	Age, smoking
Giovannucci *et al.* (1995), USA	47 894	773	Quintiles	1.1	p, trend = 0.47	1.3	p, trend = 0.004	Y	0	Age, tomato-related products, vasectomy, race
Prospective blood-based studies	No. of cases	No. of controls				Blood retinol[c]				
						RR	95% CI			
Hsing *et al.* (1990b), USA	48 (< 70 y) 55 (> 70 y)	48 55	Quartiles	–	–	0.3 0.4	p, trend = 0.08 p, trend = 0.19			Age, race
Reichman *et al.* (1990), USA	84	2356		–	–	[0.4]	[0.2–0.8]			Age
Knekt *et al.* (1990), Finland	32	59	Quintiles	–	–	[1.4]	[0.5–5.0]			Age, residence, smoking
Nomura *et al.* (1997b), USA	142	142	Quartiles	–	–	0.8	0.4–1.5			Age

[a] Study populations include both men and women unless otherwise stated

[b] Number of categories used (if quantiles not used); otherwise number of quantiles used for categorization

[c] Relative risk for highest versus lowest category

[d] Vitamin supplement use assessed (Y) or vitamin supplement use not assessed, or unclear if it was assessed (N)

no association of preformed vitamin A intake with cancer risk in northern Italy (RR in highest versus lowest tertile = 1.1, 95% CI, 0.8–1.6), but a direct association emerged for men aged 70 years or more (RR, 2.2, 95% CI, 1.1–4.2). Andersson *et al.* (1996) observed a weak positive association between retinol intake and prostate cancer. Rohan *et al.* (1995) observed a significant trend of decreasing risk of prostate cancer with increasing intake of preformed vitamin A.

Prospective data are sparse. Paganini-Hill *et al.* (1987) reported an elevation in risk for men in the highest tertile of total vitamin A, mainly attributable to increased supplemental vitamin A. In a 20-year follow-up of 17 633 white men, no overall association was observed between vitamin A intake and risk of prostate cancer (Hsing *et al.*, 1990a). An elevation in risk was apparent for men aged less than 75 years, balanced by an inverse association for men 75 years or older. Giovannucci *et al.* (1995) observed a significant positive association with retinol intake, which was stronger for men over 70 years of age.

In summary, there is no consistent evidence that dietary vitamin A protects against prostate cancer. Although initial studies suggesting an adverse effect have not been consistently confirmed, the possibility that higher intakes may increase risk requires further investigation. As most preformed vitamin A is derived from foods of animal origin, the possibility exists that other factors in animal foods may be associated with increased risk of prostate cancer, or there may be other confounding factors associated with incidence or ascertainment of prostate disease.

(h) Bladder cancer

There have been several observational epidemiological studies on the relationship between vitamin A intake and bladder cancer (see Table 16). A large hospital-based case–control study indicated lower risk associated with higher levels of total vitamin A intake (Mettlin & Graham, 1979), but the measure of vitamin A included both plant and animal sources. Subsequently, four population-based case–control studies have looked more specifically at dietary preformed vitamin A. Studies conducted in Spain (Riboli *et al.*, 1991), Hawaii (Nomura *et al.*, 1991) and Canada (Risch *et al.*, 1988) all showed no association with preformed vitamin A, but a study in the Seattle area of the United States (Bruemmer *et al.*, 1996) showed lower risk for those in the highest quintile of preformed vitamin A intake.

Taken together, the observational studies do not support the hypothesis of an association between dietary preformed vitamin A intake and the risk of bladder cancer.

(i) Cervical cancer

In a large case–control study, Marshall *et al.* (1983) observed no relation between cervical cancer risk and vitamin A derived from meats and milk (presumably mainly preformed vitamin A). In the studies of Brock *et al.* (1988), Verreault *et al.* (1989) and Herrero *et al.* (1991), little association was seen between preformed vitamin A intake and cervical cancer risk. In Milan, Italy, an index of retinol intake was not associated with invasive cervical cancer risk (La Vecchia *et al.*, 1988a). In the studies of Slattery *et al.* (1990) and Ziegler *et al.* (1990, 1991), the point estimates for high versus low consumption of total vitamin A were less than unity, but the effect was relatively small (10–20% reduction) and not statistically significant.

Potischman *et al.* (1991) analysed serum levels in the study for which Herrero *et al.* (1991) reported dietary data. A weak positive relation for serum retinol and a weak inverse relationship with β-carotene were apparent.

Overall, the available data (Table 17) suggest no relation between preformed vitamin A intake and risk of cervical cancer.

4.1.2 Intervention trials with cancer as an endpoint

4.1.2.1 Primary prevention trials

In an intervention study, each subject is allocated to one of two or more groups whose exposure is determined by the investigators. From a scientific point of view, the most reliable intervention study is one in which the allocation of exposure is carried out by randomization. Interventions which involve

Table 16. Studies of vitamin A intake and bladder cancer

Reference, site	No. of cases[a]	Categories[b]	Total vitamin A[c]		Preformed vitamin A[c]		Vit. supp.[d]	Proxies (% cases)	Adjusted for
			RR	95% CI	RR	95% CI			
Case–control studies									
Risch *et al.* (1988), Canada	826	Interquartile range	1.0	0.8–1.2	1.0	0.9–1.2	N	0	Age, smoking, diabetes
Nomura *et al.* (1991), USA	66 F 195 M		1.1 1.0	0.4–2.6 0.6–1.7	1.0 1.6	0.4–2.8 0.9–2.7	Y	0	Age, smoking
Bruemmer *et al.* (1996), USA	262	Quartiles	0.5	*p*, trend = 0.04	0.6	*p*, trend = 0.006	Y	0	Age, sex, county, smoking, calories
Riboli *et al.* (1991), Spain	432 M	Quartiles	–	–	1.3	*p*, trend = 0.30	N	0	Age, calories
Michalek *et al.* (1987), USA	102	Dichotomy	1.3	0.5–3.3		–	N	0	Age
Mettlin & Graham (1979), USA	112 F 377 M	Seven	[0.3] [0.5]	[0.1–1.1] [0.3–1.0]		–	N	0	Age
La Vecchia *et al.* (1989), Italy	163	Tertiles	–	–	0.4	*p*, trend < 0.01	N	0	Age, sex, area of residence, social class, smoking
Prospective blood-based studies	No. of cases	No. of controls			Blood retinol[c] RR	95% CI			
Helzlsouer *et al.* (1989), USA	35	70	Dichotomy	–	–	[1.3]	[0.3–5.6]		Age, smoking
Knekt *et al.* (1991b), Finland	15	29	Per S.D.	–	–	0.6	*p*, trend = 0.14		Age

[a] Study populations include both men and women unless otherwise stated

[b] Number of categories used (if quantiles not used); otherwise number of quantiles used for categorization

[c] Relative risk for highest versus lowest category

[d] Vitamin supplement use assessed (Y) or vitamin supplement use not assessed, or unclear if it was assessed (N)

Table 17. Case–control studies of vitamin A intake and invasive cervical cancer

Reference, site	No. of cases[a]	Categories[b]	Total vitamin A[c]		Preformed vitamin A[c]		Vit. supp.[d]	% Proxies (cases)	Adjusted for
			RR	95% CI	RR	95% CI			
Marshall *et al.* (1983), USA	513	Seven	–	–	1.0	*p*, trend > 0.05	N	0	Age
La Vecchia *et al.* (1988a), Italy	392	Three	–	–	1.2	0.8–1.9	N	0	Age, education, parity, body mass index, smoking, sexual behaviour, contraceptive and hormone use
Verreault *et al.* (1989), USA	189	Quartiles	–	–	1.1	*p*, trend = 0.52	Y	0	Age, education, Pap smear, energy, sexual behaviour, contraceptive use
Slattery *et al.* (1990), USA	266	Quartiles	0.9	0.5–1.6	–	–	N	0	Age, education, smoking, church attendance, sexual behaviour
Ziegler *et al.* (1990), USA	271	Quartiles	0.9	*p*, trend = 0.5	–	–	Y	0	Age, centre, Pap smears, smoking, sexual behaviour, contraceptive use, vaginal infections
Herrero *et al.* (1991), Colombia, Costa Rica, Mexico, Panama	748	Quartile	–	–	1.1	0.8–1.5	N	0	Age, centre, Pap smears, sexual behaviour, pregnancies, socioeconomc status, human papillomavirus infection

[a] Study populations include both men and women unless otherwise stated

[b] Number of categories used (if quantiles not used); otherwise number of quantiles used for categorization

[c] Relative risk for highest versus lowest category

[d] Vitamin supplement use assessed (Y) or vitamin supplement use not assessed, or unclear if it was assessed (N)

substantial dietary changes are difficult to implement, due to problems of compliance. Moreover, the possibility of bias is increased if subjects in the intervention and control groups (and also the investigators) cannot easily be kept blind to their allocation. Interventions using micronutrients, such as certain retinoids, may be easier to perform if the active treatment can be packaged in the form of an anonymous dietary supplement. However, interventions of this kind may not reflect accurately the effects of micronutrients when presented in the form of complex mixtures within foods. Issues such as optimal dosage, minimal duration, latency and adverse effects cannot always be resolved in an intervention study. A single dose is usually used to maximize study power, and the trial may have to be stopped prematurely if early advantageous or adverse effects are observed. For various reasons (such as costs, patients' compliance, disclosure of early findings, emergence of new hypotheses), most intervention trials are restricted to a few years of treatment and/or observation, thus hampering the evaluation of potential long-term benefits. On the other hand, only in an intervention trial can the effects of synthetic compounds such as synthetic retinoids be assessed.

(a) Linxian trials

Two randomized chemoprevention studies were carried out in four Linxian communes, north-central China, an area with one of the world's highest rates of oesophageal cancer (Blot *et al.*, 1993, 1995; Li *et al.*, 1993). Among several dietary deficiencies common in Linxian, riboflavin deficiency seemed the most severe.

In the first study, a total of 29 584 healthy subjects (55% females) aged 40–69 years were recruited in 1985. Using a complex factorial design, the participants were randomly assigned to seven intervention groups, which were based on four combinations of nutrients: (*a*) retinyl palmitate (5000 IU/day) and zinc; (*b*) riboflavin and niacin; (*c*) vitamin C and molybdenum; (*d*) β-carotene (15 mg/day), vitamin E and selenium, and one placebo group. Supplemented doses ranged from one to two times the United States recommended daily allowance (RDA). Participant compliance (as measured by pill disappearance and quarterly blood measurements on a random sample) was very good (>90%) and treatment groups were highly comparable in terms of sex, age, smoking, alcohol drinking and diet. The study had 90% power to detect a 23% reduction in cancer mortality during the 5.25–year supplementation period. Incident cancers and deaths were identified by several methods.

A total of 2127 deaths occurred during the intervention period (March 1986 to May 1991). Cancer was the leading cause of death, with 32% of the total mortality due to oesophageal (360 deaths) and stomach (331 deaths) cancers. Relative risks (RRs) of death and 95% confidence intervals (CI) in participants given retinol and zinc compared with non-users were 1.0 (95% CI, 0.9–1.1) for all cancers, 0.9 (95% CI, 0.8–1.2) for oesophageal cancer, and 1.0 (95% CI, 0.8–1.3) for stomach cancer (Table 18). Some protection was seen, however, for stomach cancer other than cardia (78 observed deaths, RR, 0.6; 95% CI, 0.4–0.9). RRs for cancer incidence (1298 new cancer diagnoses, RR for all cancers, 1.0; 95% CI, 0.9–1.1) were similar to the RRs for cancer deaths. Among different intervention groups, significantly lower cancer mortality (RR, 0.9; 95% CI, 0.8–1.0) occurred only among participants receiving supplementation with the combination of β-carotene, vitamin E and selenium. Blot *et al.* (1995) also reported age- and sex-specific RRs that were not significantly heterogeneous, although RRs for all cancer mortality for the retinol and zinc intervention group were lower in females than in males (0.86 and 1.07, respectively) and in participants aged <55 years than in those aged ≥55 years (0.84 and 1.03, respectively).

[The Working Group noted that the supplemental dose of retinol was relatively low (i.e., one fifth of that in most other intervention studies) and that the study design makes it impossible to distinguish the effect of retinol from that of zinc. The failure of the trial to find a significant reduction in cancer mortality among those given supplements of retinol and other compounds could be related to the shortness of the intervention and follow-up. In general, the applicability of the results to populations with adequate nutritional status and for

Table 18. Randomized chemoprevention trials including vitamin A or retinyl esters

Reference and country	Participants		Intervention(s)	Average follow-up (years)	All cancer		Specific cancers		Comments
	No.	Type			No. deaths or new cases	RR (95%CI)	Site	RR (95% CI)	
Blot *et al.* (1993), China	29 584 (55% fem.)	Healthy adults (40–69 yrs)	Factorial, including group on retinol (5000 IU/d) and zinc	5.25	792 cancer deaths	1.9 (0.9–1.1)	Oesophagus Stomach	0.9 (0.8–1.2) 1.0 (0.8–1.3)	The combination of β-carotene, vitamin E and selenium reduced cancer risk
Li *et al.* (1993), China	3318 (56% fem.)	Oesophageal dysplasia (40–69 yrs)	Multivitamin, including retinol (10 000 IU/d) versus placebo	6.0	221 new cases	1.0 (0.8–1.2)	Oesophagus Stomach	0.9 (0.7–1.2) 1.2 (0.9–1.6)	Decrease of cerebrovascular deaths in men
Omenn *et al.* (1996a,b), USA	4060 14 254 (44% fem.)	Asbestos workers Heavy smokers (mean age = 58)	Retinol (25 000 IU/d) and β-carotene (30 mg/d) versus placebo	4.0	1446 new cases	1.2 (1.0–1.3)	Lung (new cases)	1.3 (1.0–1.6)	Leukaemia RR, 2.2 (95% CI 1.0–5.0) No difference in mesotheliomas (14 in active treatment; 9 in placebo groups)
de Klerk *et al.* (1998), Western Australia	1024 (8% fem.)	Asbestos workers (median age = 57)	Retinol (25 000 IU/d) versus β-carotene (30 mg/d)	5.0	58 deaths (cancer and non-cancer)	0.6 (0.3–1.0)	Mesothelioma (3 in retinol-treated; 13 in β-carotene-treated	0.2 (0.0–0.9)	Mesothelioma rate in the retinol group was lower than among historical controls
							Lung	0.7 (0.2–2.3)	
Moon *et al.* (1997), USA	2297 (30% fem.)	Adults with ≥10 actinic keratosis (median age = 63)	Retinol (25 000 IU/d) versus placebo	3.8	115 deaths (cancer and non-cancer) 526 new skin cancers	1.1 (0.8–1.5)	Cutaneous SCC BCC	0.7 (0.6–1.0) 1.1 (0.9–1.3)	50% protection in individuals with ≥8 moles and freckles
Levine *et al.* (1997), USA	525 (29% fem.)	Adults with ≥ 4 previous skin cancers (mean age = 66)	Retinol (25 000 IU/d) or isotretinoin (5–10 mg/d), or placebo	3.8	444 new skin cancers	–	Cutaneous SCC BCC	[~1.0] [~1.0]	Hazard ratios and confidence intervals were not provided in detail

RR, relative risk; CI, confidence interval; SCC, squamous cell carcinoma; BCC, basal cell carcinoma.

cancer sites other than oesophageal and stomach may be limited.]

The other intervention trial in Linxian was carried out from May 1985 through May 1991, and included 3318 persons (56% females) aged 40–69 years, who had shown cytological evidence of oesophageal dysplasia (Li *et al.*, 1993). These diagnoses were derived primarily from population-based, oesophageal balloon cytology examinations conducted in November and December 1983. Participants were randomly assigned to supplements (one β-carotene capsule (15 mg) and two 13-vitamin, 12-mineral tablets, including retinyl acetate, 10 000 IU) or a matching placebo in a two-group design. Doses were kept at less than or equal to three times the United States RDA. Good compliance (>90%) was confirmed as in the general population trial (Blot *et al.*, 1993). Cancers were identified not only through routine surveillance, but also by special cytology and endoscopy screenings after 2.5 years and 6 years. Nearly half of all cancers were diagnosed during cytological and endoscopic screening. There were no significant differences between the randomized groups at baseline with respect to any of the subject characteristics examined (e.g., smoking, alcohol drinking and selected dietary habits), but individuals assigned to supplements had a 3% higher prevalence of grade 2 dysplasia.

No significant difference was observed for all cancer mortality (87 observed deaths in the supplement group and 89 in the placebo group, RR, 1.0; 95% CI, 0.7–1.3), oesophageal cancer (38 and 44 deaths, respectively, RR, 0.8; 95% CI, 0.5–1.3) and stomach cancer (42 and 35 deaths, respectively, RR, 1.2, 95% CI, 0.8–1.9) (Table 18). In contrast with the results reported by Blot *et al.* (1993), retinol-containing supplements were not associated with decreased mortality from non-cardia stomach cancer. Total cancer incidence was similar in the treatment and placebo groups (RR, 1.0; 95% CI, 0.8–1.2), as was incidence for oesophageal/gastric cardia cancer (RR, 1.0; 95% CI, 0.8–1.2) (Table 18). RRs of death did not vary by sex and age group (Blot *et al.*, 1995). [The Working Group noted that negative findings, according to the authors (Li *et al.*, 1993), may have been due to the shortness of intervention and follow-up and/or to supplementation delay if dysplastic lesions were no longer amenable to a benefit from nutrient supplementation. Retinol was only one of 26 vitamins and microelements supplemented in the treatment group. Furthermore, it was administered at a relatively low dose (i.e., less than half of that in other intervention trials). Finally, both studies were carried out in a period when substantial improvements were taking place in the general nutritional status of the Chinese population. This may have led to concurrent increases in the intake of vitamins and microelements in the placebo group, reducing the power of the trials to identify an effect of supplementation.]

(b) CARET

Pilot studies of the Beta-Carotene and Retinol Efficacy Trial (CARET), in the United States between 1985 and 1988, investigated 816 men with substantial occupational exposure to asbestos who received a combination of 15 mg β-carotene and 25 000 IU retinol (as palmitate) daily or placebo (1:1) and 1029 men and women with extensive cigarette-smoking histories who received 30 mg β-carotene, 25 000 IU retinol, both, or neither (2 x 2 factorial design) (Omenn *et al.*, 1996a,b). In 1988, all pilot study participants in active treatment groups were converted to the CARET efficacy regimen of 30 mg β-carotene plus 25 000 IU retinol taken daily, and the project was expanded 10-fold, including six study centres around the country during the following three years (additional randomization 1:1 active/placebo). A 2 x 2 factorial design for the efficacy trial was rejected under the hypothesis that retinol and β-carotene might have a favourable effect through complementary molecular actions (Omenn *et al.*, 1994a).

The 14 254 smokers (56% men) had a mean age of 58 years and a mean of 49 pack-years of cigarette smoking, 66% were current smokers upon recruitment (mean of 24 cigarettes per day and 48 pack-years), and 34% were former smokers (mean of three years since quitting, after smoking 28 cigarettes per day and 52 pack-years). The 4060 asbestos-exposed male workers had a mean age of 57 years; 3% were never-smokers (from the pilot study), 58% were

former smokers and 38% were current smokers, with a mean of 43 pack-years of smoking history (40 pack-years for former smokers and 47 pack-years for current smokers) and a mean of 10 years since quitting among the former smokers. They had means of 35 years since first asbestos exposure and 27-year duration of asbestos exposure on the job; approximately two thirds had positive chest X-ray results for asbestos-related disease. A net smoking cessation rate of 5% per year was achieved among study participants during the follow-up.

Up to December 1995, CARET received and validated reports of 2420 end-points, including 1446 cancers (in 1353 participants) and 974 deaths, with a median of 3.7 years and a mean of 4.0 years of follow-up after randomization for all CARET participants. Histological, immunohistochemical and ultrastructural criteria were used to diagnose the histological type of lung neoplasms. 746 additional cancer reports, which were not CARET end-points, were excluded (i.e., 500 basal cell and squamous cell skin cancers, eight cancers diagnosed before randomization, and 238 recurrences, metastatic presentations or non-cancers).

Based on a two-sided test for the primary analysis, CARET had 80% power, if carried to completion, to detect a 22% observed reduction or 24% observed increase in lung cancer incidence. The 73 135 person-years (median, 3.7) of follow-up accrued, however, corresponded to 66% of the total of 110 000 person-years projected, due to discontinuation of the trial 21 months earlier than planned.

The two randomized groups were well matched and withdrawal percentages were virtually the same in participants assigned to active treatment and those assigned to placebo (15% and 14% respectively among asbestos workers, and 20% and 19% among heavy smokers). After five years of supplementation, serum retinol levels were about 10% higher and serum β-carotene levels more than 12-fold higher in the active-treatment group than in the placebo group. There were no major side-effects attributable to the intervention regimen. The incidence of lung cancer was the primary end-point. Statistical analysis was based on stratified, weighted log-rank statistics with relative risks estimated by Cox regression models (Omenn *et al.*, 1994a).

Up to December 1995, 388 participants (2%) were reported as new cases of lung cancer (286 centrally reviewed, 254 fatal). The RR for lung cancer in the active-treatment group was 1.3 (95% CI, 1.0–1.6), with RRs of 1.4 (95% CI, 1.0–2.1) for asbestos workers, 1.4 (95% CI, 1.1–1.9) for heavy smokers who were smoking at the time of randomization, and 0.8 (95% CI, 0.5–1.3) for former heavy smokers (Table 18). Active treatment had no significant effect on the risk of mesothelioma (14 cases in the active-treatment group and 9 in the placebo group (RR, 1.5; 95% CI, 0.7–3.5) and non-lung cancer (1076 new cases, 300 of which were prostate cancers). None of several other common cancer types showed a significant difference in incidence (urinary bladder: RR, 1.1; 95% CI, 0.7–1.7; breast: RR, 0.8; 95% CI, 0.6–1.1; colorectal: RR, 1.0; 95% CI, 0.7–1.5; head and neck: RR, 1.3; 95% CI, 0.7–2.2; lymphoma: RR, 0.9; 95% CI, 0.4–2.0; prostate: RR, 1.0; 95% CI, 0.8–1.3), but a borderline elevated RR was found for leukaemia in the active-treatment group (26 observed, 2.2; 95% CI, 1.0–5.0; $p<0.06$). The RR of death from any cause in the active-treatment group was 1.2 (95% CI, 1.0–3.3) (Omenn *et al.*, 1996b).

There were suggestions that the excess lung cancer risk was associated with the highest quartile of alcohol intake (RR, 2.0; 95% CI, 1.3–3.1) and large-cell histology (RR, 1.9; 95% CI, 1.1–3.3) (Omenn *et al.*, 1996b).

[The Working Group noted that the CARET trial was based on the hypothesis that a favourable effect would come from the combination of retinyl palmitate and β-carotene. It was, therefore, unable to distinguish the individual effects of the two agents. Early discontinuation was influenced by the unexpected unfavourable effect of β-carotene in the Alpha-Tocopherol, Beta-Carotene Cancer Prevention Study (ATBC) in Finland (Cancer Prevention Study Group, 1994).]

(c) Wittenoom trial

A randomized, partially single-blind chemoprevention trial was carried out between June 1990

and May 1995 in Perth, Western Australia, among workers who had been occupationally exposed to blue asbestos (crocidolite) between 1943 and 1966 in a mine at Wittenoom Gorge (Musk *et al.*, 1998). Median duration of asbestos exposure was short (about one year). The base population for the study consisted of the 2695 members of the Wittenoom workers' cohort who have been followed for the health effects of asbestos since 1979 (de Klerk *et al.*, 1998). Out of the 1677 subjects who indicated interest, 1203 joined the study and 1024 (8% females) were randomly assigned to receive either retinyl palmitate (25 000 IU/day, $n = 512$) or β-carotene (30 mg/day, $n = 512$) (de Klerk *et al.*, 1998). The 996 members of the cohort not participating in the active intervention programme, but living within the catchment area of the death and cancer registries, formed a passive reference group.

Pill-counting and measurement of plasma β-carotene levels were performed in order to assess compliance. Deaths and incident cases of cancer were documented from the Western Australian Cancer Registry and death certificates. Follow-up was complete up to May 1995. All subjects attended the Perth Chest Clinic at least once and 48% had four annual follow-up visits. The two treatment groups were closely comparable at baseline by age (median 56 and 57 years), sex, cigarette smoking (21% current smokers, 52% ex-smokers), dietary intake and plasma levels of β-carotene (mean 6 mg, 0.5 μmol/L) and retinol (mean 0.7 mg, 2.6 μmol/L), and asbestos exposures (median 190 days of exposure, 30 years from first exposures). Comparison between groups was conducted by means of Cox regression and Fisher's exact tests in both intention-to-treat analyses and efficacy analyses (i.e., based on treatment actually taken).

Mesothelioma was the most common cause of death. After a median follow-up of 232 weeks (4½ years), the RR with retinol compared to β-carotene for mesothelioma was 0.2 (95% CI, 0.1–0.9; 15 new cases, 11 deaths) (de Klerk *et al.*, 1998). The RR from efficacy analyses was 0.1 (95% CI, 0.0–0.8) for retinol. There was no significant difference between the groups in lung cancer incidence (RR, 0.7; 95% CI, 0.2–2.3) or other cancer mortality (RR, 1.0; 95% CI, 0.2–3.9). Total mortality was significantly lower in the retinol group (21 deaths) than in the β-carotene group (37 deaths) (RR, 0.6; 95% CI, 0.3–1.0). Compared with the passive reference group, trial participants had significantly lower mortality (RR, 0.6; 95% CI, 0.5–0.9), but the rates converged with time. The incidence of both mesothelioma (RR, 0.8; 95% CI, 0.4–1.6) and lung cancer (RR, 0.7; 95% CI, 0.3–1.4) was non-significantly lower among active participants (Musk *et al.*, 1998).

The investigators concluded that there is a possibility that retinol exerts some protective effect on mesothelioma at late stages, whereas it is clear that there was no benefit from β-carotene. Since the mesothelioma rates in the retinol-treated group were only lower than rates before the start of the study among 'historical controls', the apparent benefit should not be due to a risk increase in the β-carotene-treated group (de Klerk *et al.*, 1998).

(d) Southwest Skin Cancer Prevention Studies

Two randomized, double-blind, controlled trials to examine the efficacy of retinol supplementation on the incidence of first new non-melanoma skin cancer were conducted in Arizona, United States (Moon *et al.*, 1997; Levine *et al.*, 1997).

The first included 2297 subjects (30% females), aged 21–84 years (median 63) identified through media announcements and referral to dermatologists in 1984–88. They were considered to have a moderate skin cancer risk (having a history of more than 10 actinic keratosis and at most two cutaneous squamous cell carcinomas (SCCs) or basal cell carcinomas (BCCs)) (Moon *et al.*, 1997).

All subjects were willing to limit the intake of non-study vitamin A to no more than 10 000 IU/day and were scheduled for clinic visits one month after randomization and then every six months. After a single-blind three-month placebo run-in period, the subjects were randomly allocated to receive either oral retinol (25 000 IU/day; $n = 1157$) or placebo ($n = 1140$). The groups were fully comparable according to the baseline characteristics, including an estimate of weekly sun exposure. Capsule-count adherence and serum measurements were used to evaluate compliance. Adverse symptoms were

rare (1%), but cholesterol and liver enzyme values rose for some subjects on retinol.

The study was interrupted earlier than planned for funding reasons. There were a total of 4332 study years of ascertainment period for the active treatment group and 4317 for the placebo group. Analyses of primary outcome measures were based on the Cox proportional hazards model, with adjustment for skin characteristics.

During a median follow-up time of 3.8 years, 526 subjects had a new skin cancer, 96% histologically confirmed. The active treatment group showed an RR of 0.7 (95% CI, 0.6–1.0) for cutaneous SCC (249 observed new cancers) and 1.1 (95% CI, 0.9–1.3) for BCC (417 observed new cancers), compared with the placebo group. The effect of retinol on SCC risk was consistent in different strata by age, sex, history of sun exposure and sunburn. An especially low RR, however, was found in individuals with a higher number of moles and freckles (RR, 0.5; 95% CI, 0.3–0.8). A differential (possibly lower) metabolism of retinol by basal cells compared to keratinocytes was suggested to explain the lack of benefit in terms of BCC risk (Moon *et al.*, 1997).

The second randomized, double-blind controlled trial (Levine *et al.*, 1997) included high-risk subjects (i.e., those with a history of four or more BCC or cutaneous SCC) and involved three arms: oral retinol (25 000 IU; $n = 173$), isotretinoin (5–10 mg according to weight; $n = 178$) or placebo ($n = 174$) daily for three years. Methods were similar to those of Moon *et al.* (1997). Out of 719 original participants, 525 (28% females, aged 21–85 years, median age 66 years) completed the run-in period and were randomized. Baseline characteristics, including an estimate of weekly sun exposure, and compliance were similar in the three groups. Toxicity was generally modest, but side-effects were greatest in the isotretinoin-treated group. A total of 125 SCCs and 319 BCCs were diagnosed clinically and confirmed pathologically. There were no differences between those who received the placebo and those who were given either isotretinoin or retinol with regard to time to the first occurrence of either BCC or SCC. In the retinol-treated group, the cumulative probability of cutaneous SCC at 36 months was about 5% in the study of Moon *et al.* (1997), but 28% in that of Levine *et al.* (1997). [The Working Group noted that relative risks were not reported, but similar curves of cumulative incidence were provided. However, the cumulative proportion of participants with a first new SCC seemed slightly higher in the retinol group than in the placebo groups, although not significantly so. The lack of benefit of retinol in terms of cutaneous SCC onset in high-risk individuals contrasted with findings from Moon *et al.* (1997) in moderate-risk individuals. The effects of retinoids may be more pronounced in the early stages of cancer promotion than later. Still, the lack of beneficial effect of retinol, compared with placebo, among high-risk individuals is inconsistent with the encouraging results in the companion study on moderate-risk individuals].

(e) Conclusions from the six primary prevention trials

Overall, there is no consistent evidence that four- to six-year supplementation of retinol reduces the risk of any type of cancer. In the CARET study, the risk of carcinoma of the lung was significantly elevated in the group supplemented with retinol and β-carotene compared with the placebo group, except for subjects who had stopped smoking. Among asbestos miners in the Wittenoom trial, lung cancer was less common in the retinol group than in the β-carotene group, but the difference was not statistically significant. Thus it remains possible that retinol may be less harmful than β-carotene or have no effect.

Mesothelioma, a rare neoplasm attributable to asbestos exposure, was studied in the CARET subcohort of asbestos-exposed workers and in the Wittenoom trial. In crocidolite-exposed workers in Australia, retinol intervention appeared to lead to a reduction in incidence of mesothelioma, whereas in the CARET study, where retinol was given in combination with β-carotene, no such reduction was seen. The apparent prevention of mesotheliomas in the only trial using retinol as such suggests that further studies on the effects of retinol on mesothelioma development are warranted. The two trials in Linxian (China) allowed evaluation of the effect of retinol, in combination

with either zinc or 25 additional vitamins and microelements versus placebo, especially in the prevention of cancers of the stomach and oesophagus. Cancers at these sites were extremely frequent in the study population. The two Linxian trials did not support a benefit of retinol, even in a population where nutritional deficiencies may well have existed.

Two intervention studies from the United States agreed in ruling out a benefit with respect to basal cell carcinoma of the skin. For squamous cell carcinoma, a more threatening disease than basal cell carcinoma, a risk reduction of about 25% in moderate-risk individuals is difficult to reconcile with the lack of benefit in high-risk individuals. However, the details of the end-points of the latter study have not been published.

It is worth bearing in mind that some weaknesses are largely shared by all the six trials above, making it difficult to draw firm conclusions. All studies were large, well designed and carefully conducted. However, the complexity and heavy responsibilities entailed by investigations of this size and cost led to compromises. For example, different potentially promising agents were combined in most studies, including the CARET study, and in the Wittenoom trial it was not possible to include a placebo group. All trials, except the two Linxian trials, were interrupted or modified earlier than initially planned. Intervention duration therefore never exceeded six years, a period which leaves open the issue of potential longer-term benefits of retinol were it active on relatively early stages of carcinogenesis. Finally, the choice of specific high-risk populations, though needed in order to have sufficient study power, is not without undesirable consequences with respect to the applicability of the trial results to the general population. The spectrum of cancer sites and types, for instance, was obviously heavily distorted, in all trials, to the type(s) specifically increased in the study populations. Thus, the results were largely uninformative with respect to several important cancer sites (e.g., female cancers) and shed little light on the effects of vitamin A on overall cancer mortality, particularly in women.

4.1.2.2 Prevention of second primary cancers

After epidemiological studies of vitamin A and cancer began to emerge in the 1970s, several clinical trials tested the efficacy of various types of retinoids and, less frequently, vitamin A as adjuvant therapy in patients in relation to different malignancies (Mayne & Lippman, 1997).

In a few investigations, the distinction between second primary cancers and recurrences was not made or was problematic. Both end-points are therefore shown in certain instances.

Pastorino *et al.* (1993) tested the adjuvant effect of high-dose vitamin A on 307 patients (25 females) with stage I non-small-cell lung cancer in Milan, Italy. After curative surgery, patients were randomized between 1985 and 1989 to either oral retinyl palmitate (300 000 IU orally daily for a minimum of 12 months; $n = 150$) or no treatment ($n = 157$) [no placebo was used]. After a median follow-up of 46 months, the number of patients with either recurrence or new primary tumours was 56 (37%) in the treated group and 75 (48%) in the untreated group (Table 19). Eighteen patients in the treated group developed a second primary tumour, whereas 29 patients in the control group developed 33 second primary tumours. Onset of second primaries was significantly delayed in the treated group ($p = 0.045$, log-rank test). No significant difference in overall survival emerged. In view of the only modest side-effects, it was suggested that aqueous emulsified retinyl palmitate may be less toxic than other forms of retinol. [The Working Group noted that doses of 300 000 IU per day showed limited toxicity, although daily supplements as low as 25 000 IU in other studies have produced adverse effects.]

A chemoprevention study in curatively treated patients with oral cancer, laryngeal cancer and lung cancer was started in June 1988 in Europe (EUROSCAN; de Vries *et al.*, 1991, 1993). Treatments used were aqueous emulsified retinyl palmitate (300 000 IU per day during one year and half this dose during a second year), *N*-acetylcysteine (600 mg during two years) or both drugs or neither were used, in a 2 x 2 factorial design. The last patients were entered in 1994, and the intervention period thus

Table 19. Randomized controlled cancer trials including vitamin A or retinyl esters

Reference and country	Cancer patients		Treatment	Recurrence and/or new primary tumours		Deaths		Comments
	No.	Type		Active treatment	Controls	Active treatment	Controls	
Pastorino *et al.* (1993), Italy	307 (8% female)	Stage I lung cancer	Retinol (300 000 IU/d) versus nothing	56/150	75/157	55/150	64/157	Median follow-up 46 months
				Significant delay in new primaries				
Lamm *et al.* (1994), USA	65	Bladder cancer (17% fem.)	High-versus low-dose multivitamin (retinol RDA versus 40 000 IU/d)	14/35 ($p<0.01$)	24/30	74%	76%	At 12-month follow-up
Meyskens *et al.* (1994), USA	248 (38% female)	Malignant melanoma ≥0.75 mm	Retinol (100 000 versus nothing)	Survival RR = 1.1 (95% CI, 0.7–1.7)				Median follow-up > 8 years
				Disease-free survival RR = 1.2 (95% CI, 1.8–1.8)				
Jyothirmayi *et al.* (1996), India	106 (31% female)	Head and neck cancer	Retinol (28 500 IU/d)	11/56	7/50	Not given		Median follow-up 3 years

RDA, recommended dietary allowance.

ended in 1996. In total, 2595 patients have been included, from 81 institutes in 14 different countries. Of these, 1566 (60.4%) patients had a head and neck cancer, while the other 1029 patients had been treated for lung cancer (van Zandwijk *et al.*, 1997). Of the patients receiving retinyl palmitate, 10% interrupted the treatment because of side-effects (mainly dryness and itching of the skin), but no major complication was observed. Analysis is in progress and a first report of results is expected to appear in 1998.

Lamm *et al.* (1994) compared the efficacy of two multiple vitamin regimens (i.e., RDA doses versus high doses) in diminishing recurrences of transitional cell carcinoma of the bladder in West Virginia, United States. Between 1985 and 1992, 65 such patients (11 females) were thus randomized to receive, in addition to other vitamins (B_1, B_2, B_3, B_5, B_6, B_{12}, C, D_3, E, folic acid) and zinc, either 5000 (n = 30) or 40 000 IU (n = 35) of retinyl acetate daily. After 12 months of treatment, there were 11 recurrences (37%) in the RDA group compared with 3 (9%) in the high-dose group (p = 0.008, Fisher's exact test). Overall recurrence rates were 80% (24/30) and 40% (14/35), respectively, but survival rates were similar (75% in RDA and 76% in the high-dose group) (Table 19). Due to the trial design, benefits from individual high-dose vitamins could not be distinguished (Lamm *et al.*, 1994).

A national cooperative group trial was conducted in the United States in patients with early-stage cutaneous malignant melanoma thicker than 0.75 mm, to determine if vitamin A (100 000 IU orally daily for 18 months) can increase disease-free or overall survival (Meyskens *et al.*, 1994). A total of 248 patients were randomized to vitamin A (n = 119) or observation (n = 121), with eight late exclusions. Median follow-up exceeded eight years. No differences emerged between the two groups (RR, 1.1, p = 0.71 for survival; and 1.2, p = 0.41, for disease-free survival) (Table 19). This held true for subset analyses by sex, type of other therapy, and Breslow's thickness. Overall, 12% of patients who received vitamin A experienced severe toxicity.

In Trivandrum, India, Jyothirmayi *et al.* (1996) evaluated in 1992–93 the effectiveness of vitamin A (retinyl palmitate, 28 500 IU per day orally for one year) in the prevention of local relapses and second primaries of head and neck cancer. Randomization to either vitamin A or placebo included 106 patients. Compliance was good, and no major side-effect was reported. Eleven of 56 patients [19.6%] in the vitamin A group had loco-regional recurrence compared with 5/50 [10%] in the placebo group (non-significant difference). No second primaries were observed in the vitamin A group as opposed to two in the placebo group (Table 19). The number of deaths was not reported.

One nested case–control study (Day *et al.*, 1994) in the United States investigated the possible relationship between dietary retinol intake and risk of second primary tumours in a cohort of 1090 oral and pharyngeal cancer patients. Individuals in the highest-intake quartile showed an RR of 1.6 of developing a second primary compared with individuals in the lowest quartile (p value of chi square for trend, 0.09).

In summary, of four randomized studies, only the investigation of Pastorino *et al.* (1993) showed a significant benefit from supplementation (i.e., a significant delay in new primary cancer after resection of lung cancer).

4.1.3 Preneoplastic lesions and intermediate end-points

4.1.3.1 Oral premalignancy

Stich *et al.* performed several studies on the frequency of micronuclei in cells scraped from the inside of the human cheek as a measure of chromosomal breakage (Stich *et al.*, 1982, 1984a,b, 1985, 1988a,b, 1991a,b; Stich & Rosin, 1984). Supplementation for three months in 40 Filipino betel-quid chewers with retinol (100 000 IU per week) and β-carotene (300 000 IU per week) was associated with a three-fold decrease in the mean proportion of cells with micronuclei. The frequency of micronucleated buccal mucosa cells decreased from an average of 4.2% to 1.4% (Stich *et al.*, 1984a,b; Stich & Rosin, 1984). [The Working Group noted that the study was neither randomized, blinded, nor placebo-controlled. The results were based on comparisons made in the supplemented group before and after the supplementation and not between supplemented and placebo groups.]

Stich *et al.* (1988a) later randomized fisherman from Kerala (betel-quid chewers) to placebo, β-carotene (180 mg per week) or vitamin A 100 000 IU per week and β-carotene during six months. After three months, the frequency of micronucleated cells in the group receiving vitamin A and β-carotene (n = 51) was significantly reduced from 4.0% to 1.2% in areas of leukoplakia and from 4.2% to 1.2% in normal mucosa. [The Working Group noted that the blindness of the treatment group assignment was compromised by yellowing of the oral mucosa of those participants receiving β-carotene. The results were based on comparisons made in the supplemented group before and after the supplementation and not between supplemented and placebo groups.]

In another study (Stich *et al.*, 1988b, 1991a,b), the same population participants received 200 000 IU vitamin A per week (n = 21) or placebo (n = 33) during six months. Markers under study were the number of layers of spinous cells (decrease in 85% of the participants), loss of polarity of basal cells (reduced from 72% to 22%) and subepidermal lymphocytic infiltration (diminished from 66% to 5%); nuclei with condensed chromatin disappeared from the epidermal layer (72% to 0%). The protective effect of the original treatment could be maintained for at least eight additional months by administration of lower doses of vitamin A or β-carotene. [The Working Group noted that the blindness of the study during follow-up and end-point assessment was not mentioned in the reports.]

Prasad *et al.* (1995) performed a study in reverse smokers of chutta (rolled tobacco) (n = 298). 150 subjects were randomized to receive vitamin A (10 000–25 000 twice a week for a year), riboflavin, zinc and selenium; 148 received placebo. Micronuclei and DNA adducts in the exfoliated cells of the buccal mucosa were used as markers. After 12 months, the frequency of micronuclei and the concentration of DNA adducts among those with lesions at baseline decreased significantly ($p < 0.001$) in the supplemented group compared with the placebo group. [The Working Group noted that the blindness of the study during follow-up and end-point assessment was not mentioned in the reports. Many of the reported results were based on comparisons made in the supplemented group before and after the supplementation and not between supplemented and placebo groups. In addition, micronuclei can only be regarded as a measure of DNA damage.]

Ramaswamy *et al.* (1996) studied serum levels of vitamin A in 50 patients with oral leukoplakias and 50 normal controls, 25 betel-quid chewers and 25 non-chewers, in India. A significant decrease in the serum level of vitamin A in the patients with oral leukoplakia was found, compared with the controls.

Copper *et al.* (1998) found no differences in plasma levels of the major retinoids, retinol, all-*trans*-retinoic acid, 13-*cis*-retinoic acid and 13-*cis*-4-oxoretinoic acid in 25 head and neck cancer patients from those in controls without cancer. In addition, in 10 patients from the EUROSCAN chemoprevention trial, the effect on retinoid levels of retinyl palmitate (300 000 IU/day intake during one month) was measured. The medication caused significant elevation in plasma levels of retinol (1.2-fold), all-*trans*-retinoic acid (2.2-fold), 13-*cis*-retinoic acid (5.8-fold) and 13-*cis*-4-oxoretinoic acid (8.9-fold). 13-*cis*-4-Oxoretinoic acid seems a good candidate to serve as marker to monitor compliance in future chemoprevention trials with retinol.

4.1.3.2 Reversal of oral premalignancy

Head and neck cancer (oral cancer in particular, laryngeal cancer to a lesser extent) is preceded by precancerous lesions in a considerable proportion of cases. Oral leukoplakia has been regarded as a good model to study the value of chemopreventive interventions, since the effect is directly visible and material for analysis is easily obtainable from the site of the disease. Reversal of premalignant lesions is regarded as an intermediate end-point. In many studies and chemoprevention trials, the efficacy of chemopreventive agents (retinoids, retinol and/or β-carotene) in reversing oral leukoplakia has been demonstrated (Silverman *et al.*, 1963; Raque *et al.*, 1975; Koch, 1978, 1981; Stich *et al.*, 1982, 1984a,b, 1985, 1988a,b; Shah *et al.*,

1983; Hong *et al.*, 1986; Garewal *et al.*, 1990; Toma *et al.*, 1992; Chiesa *et al.*, 1992, 1993; Lippman *et al.*, 1993; Prasad *et al.*, 1995; Sankaranarayanan *et al.*, 1997), but only in a minority was retinol the chemopreventive drug under study (Silverman *et al.*, 1963; Stich *et al.*, 1984a,b, 1988a,b; Prasad *et al.*, 1995; Sankaranarayanan *et al.*, 1997).

Silverman *et al.* (1963) were the first to administer topical vitamin A to oral leukoplakia patients, in doses of 300 000 to 900 000 IU for 1–15 weeks, with positive responses in 43%. All patients had relapses after cessation of treatment. The lower dose (300 000 IU) was as effective as the dose of 900 000 IU. [The Working Group noted the uncontrolled, open design of the study.]

Stich and co-workers have conducted a number of studies in India with betel-quid chewers, who have a high incidence of leukoplakia, using vitamin A alone or vitamin A and β-carotene together. In one study, 130 patients were randomized to placebo (n = 35), β-carotene 180 mg per week (n = 35), and β-carotene 180 mg per week with vitamin A 100 000 IU per week (n = 60) (Stich *et al.*, 1988a). At six months, complete responses were 3, 15 and 28%, respectively. New lesions were better suppressed in the combined treatment group (8%) than in the β-carotene (15%) and placebo (21%) groups. [The Working Group noted that the blindness of the treatment group assignment was compromised by yellowing of the skin of those participants receiving β-carotene.]

In a second randomized trial (Stich *et al.*,1988b), patients received placebo (n = 33) or 200 000 IU vitamin A per week (n = 21) during six months. A 57% complete remission rate and complete suppression of new lesions occurred in the treatment group, compared with a 3% remission and 21% new lesions in the placebo arm. This study population was different from that in other trials (a vitamin A deficiency cannot be excluded) and the lesions may have been caused by tobacco and betel-quid chewing. [The Working Group noted that the blindness of the study during follow-up and end-point assessment was not mentioned in the reports.]

In the study of Prasad *et al.* (1995) described in Section 4.1.3.1, complete regression of leukoplakia occurred in 57% of subjects on supplementation and 8% on placebo. [The Working Group noted that the blindness of the study during follow-up and end-point assessment was not mentioned in the reports. The reported results were based on comparisons made in the supplemented group before and after the supplementation and not between supplemented and placebo groups.]

Sankaranarayanan *et al.* (1997) conducted a double-blind placebo-controlled trial to evaluate the chemopreventive potential of either retinol alone or β-carotene alone in subjects with oral leukoplakia in Kerala, India. Fishermen and women with oral precancerous lesions (n = 160) were randomized to receive oral vitamin A (retinyl acetate 300 000 IU per week, n = 50), or β-carotene (360 mg per week, n = 55) or placebo (n = 55) for 12 months. The results were based on 43 compliant subjects on placebo, 42 on vitamin A and 46 on β-carotene. The complete regression rates were: [7%] in the placebo arm, 52% with vitamin A ($p < 0.0001$) and [33%] with β-carotene ($p < 0.05$). Half of the responders with β-carotene and two thirds of those with vitamin A relapsed after treatment. [The Working Group noted the use of only 'compliant' participants and the rejection of the intention-to-treat principle.]

In conclusion, retinol seems to be active in oral leukoplakia. Recurrences occur after treatment. The meaning of these findings remains unclear, in particular as to how far these results can be extrapolated to overt oral cancer.

4.1.3.3 Larynx

Issing *et al.* (1997) performed a study of laryngeal squamous cell hyperplasia using retinyl palmitate in a induction-phase dose of 300 000 IU daily, increasing in case of resistant lesions and with a later maintenance phase of 150 000 IU/day. Fifteen out of 20 cases showed complete response, while in the other five patients partial remission was seen.

4.1.3.4 Oesophagus and stomach

A randomized double-blind intervention trial was carried out in Huixian, China, to determine

whether combined treatment with retinol, riboflavin and zinc could lower the prevalence of precancerous lesions of the oesophagus. Subjects received either retinol (50 000 IU), 200 mg riboflavin and 50 mg zinc once a week (n = 305) or placebo (n = 305) (Muñoz *et al.*, 1985, 1987a). The intervention did not affect the prevalence of oesophageal lesions; after one year, the prevalence of oesophagitis with or without atrophy or dysplasia was 45.3% in the placebo group and 48.9% in the vitamin/zinc group. Significant site-specific suppression of micronuclei (oesophageal but not buccal) was observed in treated subjects. However, the plasma retinol levels increased substantially in both treatment and placebo groups (Thurnham *et al.*, 1988). This may have been due to social changes taking place in China at that time, although there was no change in riboflavin status in the placebo group. The data were therefore re-analysed using logistic regression analysis in which all data were combined and the prevalence of oesophageal lesions was shown to be significantly lower in those whose retinol increased over the years (Wahrendorf *et al.*, 1988). Thus, improvement in vitamin A status may have reduced the inflammatory lesions in the oesophagus.

Wang *et al.* (1994) reported on a randomized intervention trial among 29 504 residents of Linxian, China [Linxian General Population Study, see also Section 4.1.2.1(*a*) and Blot *et al.* (1993)]. A fractional factorial study design allowed evaluation of four different combinations of nutrients: (*a*) retinol and zinc, (*b*) riboflavin and niacin, (*c*) vitamin C and molybdenum, and (*d*) β-carotene, vitamin E and selenium, in seven groups including placebo. At the end of the 5.25-year intervention, endoscopy was performed on 391 individuals. A reduction in risk (RR, 0.38, p = 0.09) was seen for the effect of retinol and zinc on the prevalence of gastric cancer. Oesophageal biopsy results showed no convincing evidence that any of the supplement combinations decreased the prevalence of oesophageal dysplasia.

In a cross-sectional study of serum micronutrient levels and prevalent dysplasia, subjects with dysplasia had lower carotene levels, but equivalent retinol levels, to controls (Haenszel *et al.*, 1985). In a similar study in China, serum levels of β-carotene were lower among subjects with intestinal metaplasia or dysplasia than among controls; retinol levels were equivalent (Zhang *et al.*, 1994).

4.1.3.5 Colorectum

Nearly all colorectal cancers arise from adenomas which are polypoid neoplasms usually present for years before progression to invasive cancer. Several epidemiological studies have examined nutritional factors in colorectal adenomas (see Potter, 1996, for review). Two case–control studies have presented results for vitamin A. A study in Denmark found lower risk with higher intake of dietary vitamin A (RR, 0.7; 95% CI, 0.4–1.1 for highest tertile) (Olsen *et al.*, 1994). However, a study in Spain found a similar association with total vitamin A (RR, 0.5 for highest quartile, p, trend < 0.05), but no association with retinol (RR, 1.0 for highest quartile, p, trend > 0.05) (Benito *et al.*, 1993). This limited evidence would support a protective role for pre-vitamin A compounds in fruits and vegetables, but not any association specific to retinol in colorectal adenomas.

4.1.3.6 Lung

A randomized, placebo-controlled clinical trial of β-carotene and retinol was conducted among 755 former asbestos workers in Texas, United States (McLarty *et al.*, 1995). The targeted endpoint for the intervention study was a reduction in the incidence and prevalence of sputum atypia. The dosage of 50 mg β-carotene per day and 25 000 IU retinol per day on alternate days resulted in significant increases in serum concentrations of both agents with no significant clinical toxicity. No significant reduction in sputum atypia was observed after treatment compared with placebo.

4.1.3.7 Cervical dysplasia

Lambert *et al.* (1981) compared serum levels of vitamin A and carotene in 14 patients with cervical intraepithelial neoplasia (CIN) with those in 10 controls, in order to study a possible vitamin A deficiency as a causative factor in malignant transformation of cervical metaplasia. No

significant vitamin A deficiency was found in these patients.

Brock *et al.* (1988a) investigated blood and dietary measures of vitamin A status in 117 patients with *in situ* cervical cancer and 196 matched controls. Neither retinol from food nor plasma retinol was related to cancer risk.

La Vecchia *et al.* (1988a) analysed 392 cases of invasive cervical cancer compared with 392 age-matched controls, and 247 cases of CIN compared with 247 controls with normal smears. No association emerged between any of the food items containing vitamin A and CIN.

Cuzick *et al.* (1990) investigated serum vitamin A and vitamin E levels in women aged 16–40 years, in a case–control study of CIN. The findings showed no association between serum vitamin A levels and cervical neoplasia.

Ziegler *et al.* (1991) performed a case–control study of 229 women with *in situ* cervical cancer and 502 controls. Vitamin A intake was unrelated to the risk of *in situ* cervical cancer.

De Vet *et al.* (1991) performed a case–control study of the effects of β-carotene and several other dietary factors on the risk of cervical dysplasia. Cases (n = 257) were the participants in a randomized trial assessing the effect of β-carotene on cervical dysplasia, and controls (n = 705) were sampled from the general population. An increased risk of cervical dysplasia was observed for women with a high intake of β-carotene, but no association was found with the intake of retinol.

Liu *et al.* (1995) evaluated the effect of human papillomavirus type 16 (HPV-16) infection and nutritional status on the course of cervical dysplasia in 206 women. HPV-16 infection was found to be related to the progression of cervical dysplasia, with a relative risk of 1.2. High plasma levels of retinol were related to the regression of cervical dysplasia, especially in HPV-16-positive women. Compared with women having plasma retinol levels below 0.45 μg/mL, the relative risk was 0.7 for those with retinol levels of 0.45–0.61 μg/mL, and 0.7 for those with retinol levels above 0.61 μg/mL.

Buckley *et al.* (1992) found no difference in retinol intake between American Indian women with cervical dysplasia (n = 42) and women with normal cervical cytology (n = 58).

Palan *et al.* (1996) investigated plasma levels of β-carotene, lycopene, canthaxanthin, retinol, and α- and τ-tocopherol. The target population of 235 included women with histopathological diagnosis of CIN or cervical cancer and a control group. The mean plasma level of carotenoids as well as that of α-tocopherol were significantly lower in women with CIN and cervical cancer. The mean level of τ-tocopherol was higher among patients with CIN, while the mean plasma levels of retinol were similar among the groups.

4.1.3.8 Systemic biomarkers

The relationships between plasma levels or dietary intake of retinol and levels of carcinogen–DNA adducts have been investigated. Mooney *et al.* (1997) evaluated DNA adducts with polycyclic aromatic hydrocarbons by competitive ELISA with fluorescence detection in leukocytes of 159 heavy smokers, and found an inverse association with their plasma levels of retinol. Wiencke *et al.* (1995) measured smoke-related DNA adducts by ^{32}P-postlabelling analysis in blood mononuclear cells and lung tissue obtained at surgery from 31 lung cancer patients. DNA adducts in both blood and lung were inversely related to the dietary intake of retinol.

Urinary excretion of aflatoxin B_1–DNA adducts in healthy Taiwanese males was positively associated with plasma levels of α- and β-carotene, but no association of the adducts levels with plasma levels of retinol was found (Yu *et al.*, 1997).

The incidence of chromosomal aberrations in cultured lymphocytes of 109 styrene-, formaldehyde- and phenol-exposed workers was compared with that of 64 controls. There was a marked increase in the incidence of the structural chromosomal aberrations in the first mitotic division metaphases of the occupationally exposed workers. 22 occupationally exposed workers were selected for a trial including one month's administration of capsules with retinyl palmitate 100 000 IU plus 0.1 mg α-tocopherol acetate dissolved in 0.2 mL of oil at a dose of 1–2 capsules for five days per week. The drug combination caused a significant decrease in occupationally induced chromosomal damage in lymphocytes (Mierauskienè *et al.*, 1993).

4.1.3.9 Summary

In conclusion, studies using biomarkers as intermediate end-points have been performed in relation to oral leukoplakia and oesophageal dysplasia. Although changes in expression were found in some instances, their relevance remains to be established.

Of the premalignant lesions, oral leukoplakia is the best studied. Studies performed in developing countries have shown positive results, but it remains unclear how far these results can be extrapolated to other populations.

4.2 Experimental models

4.2.1 Tumour induction

In the following section, studies evaluating effects of retinol and retinyl esters on experimental carcinogenesis are described. Several tumour models have been used to study the effects of vitamin A and its analogues on the development of cancers. These have largely used chemical carcinogens for induction of target organ-specific cancers. For example, 7,12-dimethylbenz[*a*]anthracene (DMBA) and *N*-methyl-*N*-nitrosourea (MNU) were used for induction of breast cancer, whereas *N*-[4-(5-nitro-2-furyl)-2-thiazolyl]formamide (FANFT) was used for induction of urinary bladder cancer. It should be noted that the majority of carcinogenesis studies are carried out at a high carcinogen concentration and the modulatory chemopreventive agents are used as pharmacological doses rather than physiological concentrations. This poses problems in correlating the results with those derived from clinical trials. Secondly, the mammary carcinogenesis studies were conducted with young animals and therefore may not model closely the effects of vitamin A on breast cancer in post-menopausal women.

Several further studies have been reported evaluating effects of vitamin A and esters, but were inadequately designed. Such studies were not included in this section. Effects of vitamin A and esters on the development of spontaneous tumours are described in Section 6.

4.2.1.1 Lung (Table 20)

(a) Rat

Over 230 Fischer 344 rats (males and females; 15–22 rats per subgroup [not further specified]) were placed on a semisynthetic vitamin A-free diet (TD-69389, General Biochemicals) at three weeks of age. One week thereafter, they were divided into three groups which received retinyl acetate twice weekly by gavage amounting to weekly doses of 17.4, 174 or 1740 mg retinyl acetate per rat. Five weeks later, the animals were treated with two intratracheal instillations (on consecutive days) of 3-methylcholanthrene (MCA); the total dose amounted to 1.25, 2.5, 5 or 10 mg of carcinogen, providing four groups of rats for each level of retinyl acetate. The animals were killed when moribund or at 180 weeks after carcinogen treatment. Pulmonary squamous-cell cancer incidence was higher among rats at a low dose of retinyl acetate (17.4 mg/week/rat) in comparison with rats given the highest retinyl acetate supplement. This phenomenon was observed at all four dose levels of carcinogen: 10 mg (93% versus 66%), 5 mg (65% versus 20%), 2.5 mg (27% versus 9%) and 1.25 mg (23% versus 10%) (Nettesheim *et al.*, 1979).

(b) Hamster

Two groups of 36 male and 36 female Syrian golden hamsters, 12 weeks of age, were given intratracheal instillations of benzo[*a*]pyrene (BP) adsorbed onto ferric oxide particles (3 mg BP + 3 mg ferric oxide in 0.2 mL saline) once a week for 10 weeks. Group 1 received no further treatment. Group 2 received retinyl palmitate (5000 IU in 0.1 mL corn oil) by stomach tube twice weekly for life starting seven days after the last intratracheal instillation. The incidence of respiratory tract tumours (tracheal and bronchial squamous-cell papillomas and carcinomas combined) was 11/53 in the BP-only group versus 1/46 in the group given BP plus retinyl palmitate. It was suggested that retinyl palmitate has a systemic inhibitory effect on the development of metaplastic and benign and malignant neoplastic lesions in the respiratory tract of BP-treated hamsters (Saffiotti *et al.*, 1967). [The Working Group noted that this was a pilot study without statistical evaluation of the data.]

In a similar experiment, the effects of intragastrically administered retinyl acetate on lung tumour development in hamsters induced by

Table 20. Effects of vitamin A and retinyl esters on lung tumorigenesis

Species, strain, sex, age	No. of animals	Carcinogen (dose/route)	Retinoid (dose/route)	Treatment relative to carcinogen	Preventive efficacy[a]	Reference
Rat, Fischer 344, male and female, 3 weeks old	230 rats (15–22 rats/group)	3-MCA, intratracheally 0.5 dose × 2 times (total dose, 1.3, 2.5, 5 or 12 mg/rat)	Retinyl acetate, 17.4, 174 or 1740 μg/rat/week given by stomach tube; 0.5 dose 2 times/week for up to 180 weeks	Before and during	Effective at all doses of carcinogen	Nettesheim *et al.* (1979)
Hamster, Syrian golden, male and female, 12 weeks old	36 males and 36 females per group	BP (3 mg) + ferric oxide (3 mg), intratracheal instillation weekly for 10 weeks	5000 IU retinyl palmitate in 0.1 mL corn oil twice weekly, intragastrically for life	After	Effective	Saffiotti *et al.* (1967)
Hamster, Syrian golden, male, 12 weeks old	83 74 73	12 × 3 mg BP 12 × 3 mg BP 12 × 3 mg BP	Retinyl acetate 100 μg/week 1600 μg/week 2400 μg/week, intragastrically for life	After	Ineffective	Smith *et al.* (1975a)
Hamster, Syrian golden, male, 12 weeks old	109 111 107	12 × 3 mg BP 12 × 3 mg BP 12 × 3 mg BP	Retinyl acetate 100 μg/week 1600 μg/week 2400 μg/week for life	After	Ineffective	Smith *et al.* (1975b)

Abbreviations: BP, benzo[*a*]pyrene; MCA, 3-methylcholanthrene

[a] Effective implies a statistically significant inhibition

BP adsorbed onto ferric oxide particles (12 weekly intratracheal instillations) were studied. One week after the last carcinogen administration, the hamsters, which were fed a commercial diet, were divided into three groups receiving either 100 μg (Group 1), 1600 μg (Group 2) or 3300 μg (Group 3; later reduced to 2400 μg) retinyl acetate intragastrically in cotton seed oil for life [a control group not receiving retinyl acetate was not included]. In Group 1, 48/83 animals (58%) had benign and malignant respiratory tract tumours combined (72 tumours total); in Group 2, 52/74 animals (70%) had respiratory tract tumours (70 tumours in total); in Group 3, 59/73 animals (81%) had respiratory tract tumours (84 tumours in total). The increase in the tumour incidence in Group 3 versus Group 1 was statistically significant ($p < 0.01$). However, the incidence of squamous-cell carcinomas and adenocarcinomas in the respiratory tract was similar in all three groups (Smith *et al.*, 1975a).

Similar results were obtained by Smith *et al.* (1975b). Hamsters were exposed to the same BP/ferric oxide mixture and treated with the same retinyl acetate doses and were maintained on a semi-synthetic diet and kept in conventional housing or in laminar flow units (to reduce respiratory infections). The incidence of respiratory tract tumours was not statistically significantly different between the low- and high-dose retinyl acetate groups. It was noted, however, that the proportions of hamsters with stomach papillomas were significantly reduced ($p < 0.005$) in the two high-dose retinyl acetate groups (1600 μg and 2400 μg retinyl acetate, respectively) compared with the low-dose retinyl acetate group (100 μg retinyl acetate), namely 25% and 26% with papillomas, respectively, compared with 50%.

4.2.1.2 Mammary gland (Table 21)

All of the following studies were conducted in young adult animals.

(a) Mouse

Groups of 20–30 female C3H/A mice were fed retinyl acetate in the diet starting either at conception, as weanlings or at three months of age. The groups received retinyl acetate at concentrations of either 21, 41, 83, 166 or 333 mg/kg of diet in the form of gelatin beadlets. No significant difference in the incidence of mammary carcinomas was found between controls and retinyl acetate-fed mice. The incidence in experimental groups of mice varied from 80 to 92%. The number of tumours per mouse and the tumour latency period were also not influenced by retinyl acetate in the diet (Maiorana & Gullino, 1980).

Forty-day-old GR/A female mice were treated with estrone (0.5 mg/L in the drinking-water) and progesterone (50 mg subcutaneous pellet made with 10 mg cholesterol). Animals were fed either basal diet (not specified) or diet containing retinyl acetate at a concentration of 82 mg/kg of diet in the form of beadlets for 13–14 weeks, at which time the study was terminated. The incidence of mammary tumours [not examined histologically] was 22/65 (34%) in controls compared with 37/65 (57%) ($p < 0.05$) in retinyl acetate-treated mice. This enhancement of tumour development was recorded for both nulliparous and multiparous mice (Welsch *et al.*, 1981).

(b) Rat

Groups of 50 female Sprague–Dawley rats were treated with a single intragastric dose of either 2.5, 5 or 15 mg DMBA in 1 mL sesame oil. Retinyl acetate treatment (1 or 2.5 mg/rat per day in the form of gelatin beadlets mixed in the diet) was initiated seven days after carcinogen treatment for 211 days. All animals were maintained on conventional diets. There was only 8% incidence of histopathologically confirmed mammary carcinomas in the 2.5 mg DMBA control group of rats. At 5 and 15 mg DMBA, the incidence of mammary carcinomas was 46 and 75%, respectively. Retinyl acetate was ineffective at the 1 mg dose regardless of DMBA dose; however, at 2.5 mg retinyl acetate dose, there was a significant ($p < 0.05$) decrease in the incidence of adenocarcinomas (11/50 compared with 23/50 in controls) and of adenomas (30/50 compared with 43/50 in controls) (Moon *et al.*, 1976).

Groups of 30 female Sprague-Dawley rats, 50 days of age, were injected twice intravenously

Table 21. Effects of vitamin A and retinyl esters on breast tumorigenesis

Species, strain, sex, age	No. of animals	Carcinogen (dose/route)	Retinoid (dose/route)	Treatment relative to carcinogen	Preventive efficacy	Reference
Mouse, C3H/A, female, 3 weeks old	Control 102, experimental 20–30/group	Murine mammary tumour virus positive	Retinyl acetate, 21, 41, 83, 166 or 333 mg/kg of diet	From conception, from weanling or from 3 months	Ineffective	Maiorana & Gullino (1980)
Mouse, GR/A, females, 40 days old	65/group	Estrone + progesterone	Retinyl acetate, 82 mg/kg diet for 13–14 weeks	Before, during and after	Increased tumour incidence	Welsch *et al.* (1981)
Rat, Sprague-Dawley, females, 50 days old	50/group	DMBA: 2.5, 5 or 15 mg	Retinyl acetate: 1 or 2.5 mg per day in diet for 211 days	After	Ineffective at 1 mg. Effective at 2.5 mg	Moon *et al.* (1976)
Rat, Sprague-Dawley, females, 50 days old	30/group	MNU: 12.5, 25 or 50 mg/kg bw intravenous injection × 2	Retinyl acetate: 250 ppm in the diet	Before, during and after	Effective at all doses of carcinogen	Moon *et al.* (1977)
Lewis rats, females, 50 days old	20/group	DMBA: 20 mg intragastrically at 50 days of age	Retinyl acetate: 250 mg/kg of diet	(a) Before and during (b) After, 1–30 weeks (c) After, 1–2 weeks (d) After, 12–30 weeks (e) Before, during and after	Effective Effective Effective Effective Effective	McCormick *et al.* (1980)
Rat, ACI, female 59–65 days old	24/group	17_-Ethinylestradiol pellet (1 mg/pellet)	Retinyl acetate 420 IU/g diet + 4 IU retinyl palmitate/g diet for 25 weeks	Before and during	Effective	Holtzman (1988)

Table 21 (contd)

Species, strain, sex, age	No. of animals	Carcinogen (dose/route)	Retinoid (dose/route)	Treatment relative to carcinogen	Preventive efficacy	Reference
Rat, Lew/Mai females, 50 days old	20/group	BP: 50 mg single dose intragastrically	Retinyl acetate: 250 mg/kg diet for up to 90 weeks	(a) Before and during (b) After and during, up to 90 weeks (c) After and during, up to 20 weeks (d) After, 20–35 weeks –2 to +90 weeks	Effective Effective Ineffective Effective Effective Effective	McCormick *et al.* (1981)
		6.25 mg/wk/8 wks				
Rat, Sprague-Dawley, females, 50 days old	25/group	DMBA: 15–20 mg single dose intragastrically	Retinyl acetate: 328 mg/kg diet for 240 days	+1 to + 17 weeks	Effective	Thompson *et al.* (1982)
Rat, Sprague-Dawley, females, 50 days old	30/group	MNU: 50 mg/kg bw single dose intravenously	Retinyl acetate: 328 mg/kg diet (duration not given)	+1 to + 17 weeks	Effective	Moon *et al.* (1983)
Rat, Sprague-Dawley, females, 50 days old	30/group	DMBA: 16 mg single dose intragastrically	Retinyl acetate: 250 mg/kg diet	Before and during After Before, during and after	Ineffective Effective Effective	McCormick *et al.* (1986)
Rat, Sprague-Dawley, females, 50 days old	30/group	DMBA: 5 mg intragastrically, 6 times	Retinyl acetate: i.p. injection once weekly at 350 mg/kg bw for 50 days; 250 mg/kg bw for 90 days; and 200 mg/kg bw for 60 days until 240 days of age	Before and during	Effective	Ramesha *et al.* (1990)
Rat, Holtzman, female, 50 days old	20/group	DMBA: 20 mg single dose intragastrically	Retinyl acetate 50 mg/kg diet	Before, during and after, up to 10 days	Ineffective	Rao *et al.* (1990)

Abbreviations: BP, benzo[*a*]pyrene; DMBA, 7,12-dimethylbenz[*a*]anthracene; MNU, *N*-methyl-*N*-nitrosourea

with either 12.5, 25 or 50 mg/kg bw MNU. The injections were given one week apart. Retinyl acetate in the form of gelatin beadlets was mixed with the Purina Lab chow diet at a concentration of 0 or 250 mg/kg of diet. The treatment was started one week after initiation and continued for 175 days. Retinyl acetate inhibited tumour incidence significantly compared with groups treated with carcinogen alone. The incidence of mammary tumour-bearing (retinyl acetate-treated versus untreated) was: 15/30 versus 25/30 in the high-dose MNU group, 7/30 versus 12/29 in the mid-dose MNU group and 0/30 versus 6/30 in the low-dose MNU group. At the high dose, control rats developed 61 cancers compared with 25 cancers in the retinyl acetate-treated group (Moon *et al.*, 1977).

Groups of 20 female Lewis rats, 50 days of age, were treated with 20 mg DMBA in 1 mL sesame oil. Retinyl acetate in gelatin beadlets was mixed with Purina Lab chow at a concentration of 250 mg/kg of diet. In relation to the time of DMBA treatment, rats were given retinyl acetate during weeks –2 to +1, –2 to +30, +1 to +30, +1 to +12 or +12 to +30. The experiment was terminated at 30 weeks. The greatest inhibition of mammary tumour yield was observed in the group receiving retinyl acetate for the longest time (–2 to +30 weeks); mammary tumour multiplicity was approximately 60% of that in the controls. A similar inhibition of 60% was also reported after a shorter exposure to retinyl acetate of –2 to +1 week (McCormick *et al.*, 1980). [The Working Group noted that the mammary tumours were not examined histologically and that data were presented only as graphs.]

McCormick and Moon (1982) studied the effect of delaying retinyl acetate treatment after MNU administration. All experimental details were the same as described by Moon *et al.* (1977), except that a single injection of 25 mg/kg bw MNU was given. Treatment with retinyl acetate at a concentration of 328 mg/kg of diet was initiated 1, 4, 8, 12, 16 or 20 weeks after the carcinogen treatment. The results showed that the treatment with retinyl acetate could be delayed for as long as 12 weeks without loss of efficacy.

Groups of 20 Lew/Mai rats, 50 days of age, received either a single dose of 50 mg BP in 1 mL sesame oil intragastrically or eight weekly doses of 6.25 mg BP. Rats were given Purina Lab chow as control diet or a diet containing 250 mg/kg of diet retinyl acetate in the form of gelatin beadlets. In relation to the time of BP treatment, retinyl acetate was given during weeks –2 to +1, +1 to +90, +1 to +20 or +20 to +90. Animals were weighed monthly and killed 90 weeks after the carcinogen treatment. Rats given only the single injection of BP had a 77% incidence of mammary tumours. The incidence in the retinyl acetate-treated groups was: –2 to +1, 44% ($p < 0.01$); +1 to +90, 42% ($p < 0.01$); +1 to +20, 71%; and +20 to +90, 45% ($p < 0.01$). There was a 67% incidence of mammary tumours in animals given eight injections of BP alone; retinyl acetate given from –2 to +90 weeks reduced this to 40% ($p < 0.01$) (McCormick *et al.*, 1981). [The Working Group noted that the tumours were not evaluated histopathologically.]

Groups of 25 Sprague–Dawley rats, 50 days of age, received 20 mg DMBA intragastrically and were ovariectomized at various times in relation to carcinogen treatment. Intact and ovariectomized animals were treated with retinyl acetate at a concentration of 328 mg/kg of diet. The incidence of mammary adenocarcinomas was 10/25 in ovariectomized rats compared with 4/25 in the rats that were ovariectomized and received retinyl acetate treatment. Tumour multiplicity in this study was also reduced by retinyl acetate (Thompson *et al.*, 1982).

Four groups of 30 female Sprague–Dawley rats were treated with 50 mg/kg bw MNU by intravenous injection. Rats were either kept intact or were ovariectomized 14 days after carcinogen treatment. Both intact and ovariectomized rats either served as separate controls or received 1 mmol retinyl acetate/kg of diet [328 mg/kg of diet] in the form of gelatin beadlets. Although retinyl acetate reduced the incidence of mammary adenocarcinomas from 80% in controls to 59% in the retinyl acetate-treated group, ovariectomy reduced the incidence to 35%. The combination of ovariectomy and retinyl acetate resulted in a tumour incidence of less than 10%. This remarkable suppression of carcinogenesis was accompanied by

increased latency of tumour development from 30–50 days in controls to 150 days in the combined treatment group (Moon *et al.*, 1983). [The Working Group noted that data were presented only as graphs.]

McCormick *et al.* (1986) studied the effect of combined treatment with retinyl acetate and butylated hydroxytoluene (BHT) in groups of 30 female Sprague–Dawley rats treated with 16 mg of DMBA in 1 mL sesame oil at 50 days of age. Animals were given either 250 mg retinyl acetate/kg of diet as gelatin beadlets alone or in combination with BHT at a concentration of 5 g/kg of diet. In relation to the time of DMBA treatment, retinyl acetate/BHT was given during weeks –2 to +1, +1 to + 26, or –2 to + 26 (180 days). Body weights were not affected in these studies. The multiplicity of mammary carcinomas was reduced from 7.5 tumours per rat in controls to 4.97 and 4.74 following treatment with retinyl acetate from + 1 to + 26 and from –2 to + 26 weeks, respectively (66% and 63% of controls, respectively). The combination of BHT and retinyl acetate was more effective than either treatment alone.

Two groups of 30 Sprague–Dawley rats, 40 days of age, were treated with 5 mg DMBA in sesame oil per week for six weeks (total dose, 30 mg). Retinyl acetate was injected intraperitoneally once weekly at doses of 350 mg/kg bw (40 days), 250 mg/kg bw (90 days) and 200 mg/kg bw (60 days) until the animals reached the age of 240 days. Retinyl acetate significantly reduced the numbers of of tumour-bearing animals from 28/28 in DMBA-treated controls to 13/28 ($p < 0.001$). When retinyl acetate treatment was combined with other compounds such as sodium selenite, magnesium chloride and ascorbic acid, the inhibitory effect of retinyl acetate was enhanced (Ramesha *et al.*, 1990).

Female Holtzman rats were treated with 20 mg DMBA in 0.5 mL oil. They were given either control diet or diet mixed with retinyl acetate (50 mg/kg of diet) alone or in combination with tamoxifen, tocopherol, aminoglutethimide, ergocryptine and/or selenium. The chemopreventive agent in the diet was given for 21 days beginning 10 days before carcinogen treatment and continuing to 10 days after carcinogen treatment. Rats were killed 180 days after carcinogen treatment and the tumours were examined histopathologically. Mammary adenomas developed in 10/15 DMBA-exposed rats and in 12/20 rats treated with DMBA plus retinyl acetate. When retinyl acetate was given in combination with two or more other agents, the tumour incidence was reduced from 64% to 20–30% ($p < 0.05$) (Rao *et al.*, 1990).

Two groups of female ACI rats were divided into 24 rats per group. All animals received AIN-16A semisynthetic diet with 4000 IU of retinyl palmitate. The active treatment group had its diet supplemented with 412 000 IU of retinyl acetate per kg diet. After two weeks, subcutaneous pellets of 1 mg 17α-ethinylestradiol were implanted into both groups of rats. Animals were killed 25 weeks later. Retinyl acetate treatment did not affect body weight. The incidence of mammary carcinomas was 88% (21/24) in controls rats and 70% (16/23) in the retinyl acetate-treated group ($p < 0.05$). The multiplicity was reduced from 5.6 cancers per rat in controls to 2.7 cancers per rat in the retinyl acetate-treated group ($p < 0.05$) (Holtzman, 1988).

4.2.1.3 Urinary bladder (Table 22)

(a) Rat

Three groups of 40 weanling female Sprague–Dawley rats, weighing 44 to 76 g, were fed vitamin A-deficient diets and received either (1) 100 IU retinyl palmitate via stomach tube during weeks 9 and 16; (2) 5 IU retinyl palmitate/g diet or (3) 250 IU retinyl palmitate/g diet for the first four weeks and 500 IU/g diet thereafter. After one week, 0.188% FANFT was added to these diets for 12 weeks and 0.1% for the following eight weeks. At 22 weeks, the incidences of bladder carcinomas (transitional-cell and squamous-cell) were 21/40, 17/40 and 16/40 in the three groups, respectively (Cohen *et al.*, 1976).

In a second experiment, two groups of 36 weanling female Sprague–Dawley rats, weighing 44 to 76 g, were fed a basal diet either with or without added retinyl palmitate (930 up to 2500 IU/g diet). After one week on these diets, 0.1% FANFT was added to the diet for 20 weeks, after which the rats received their respective

Table 22. Chemopreventive activity of vitamin A and retinyl esters on urinary bladder carcinogenesis

Species, strain, sex	No. of animals	Carcinogen, dose, route and duration of administration	Retinoid (dose/route)	Treatment relative to carcinogen	Preventive efficacy	Reference
Rat, Sprague-Dawley, female, 3–4 weeks old	40/group	*N*-4-(5-Nitro-2-furyl)-2-thiazolyl formamide in the diet; 0.188% for 12 weeks and 0.1% for the next 8 weeks	Retinyl palmitate, 0, 5, 250, 500 IU per g diet for 22 weeks	Before and during	Ineffective	Cohen *et al.* (1976)
Rat, Sprague-Dawley, female, 3–4 weeks old	36/group	*N*-4-(5-Nitro-2-furyl)-2-thiazolyl formamide in the diet (0.1%) for 20 weeks	Retinyl palmitate, 0, 930, 2500, 1100 IU per g diet for 35 weeks	Before, during and after	Ineffective	Cohen *et al.* (1976)
Rat, Wistar, male, weight 165 g	18/group	*N*-Butyl-*N*-(4-hydroxybutyl)-nitrosamine via drinking water, 0, 0.01, 0.025% for 20 weeks	Retinyl acetate, 0, 100, 200 IU per g diet for 20 weeks	During	Effective at 200 IU	Miyata *et al.* (1978)

diets without FANFT. All rats alive at 35 weeks were killed. Signs of hypervitaminosis A occurred by 11 weeks in the group maintained on 2500 IU/g diet (fed in weeks 6–13). Therefore the concentration was reduced to 1100 IU/g diet after 13 weeks. The incidence of transitional-cell carcinomas (2/36 in both groups) was similar in the group fed normal vitamin A diet and in the group on the retinyl palmitate-supplemented diet (Cohen *et al.*, 1976).

Nine groups of 18 male Wistar rats, weighing approximately 165 g, were treated simultaneously with *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine (BBN) in the drinking water and/or retinyl acetate in the diet for 20 weeks as follows: (1) 200 IU retinyl acetate/g diet and 0.025% BBN; (2) 100 IU retinyl acetate/g diet and 0.025% BBN; (3) 0.025% BBN; (4) 200 IU retinyl acetate/g diet and 0.01% BBN; (5) 100 IU retinyl acetate/g diet and 0.01% BBN; (6) 0.01% BBN; (7) 200 IU retinyl acetate/g diet; (8) 100 IU retinyl acetate/g diet and (9) controls, with 10 IU of retinyl acetate in basal diet. All surviving rats were killed after 20 weeks. Administration of 200 IU retinyl acetate/g diet reduced the incidence of transitional cell papillomas (1/18 vs 7/16) and carcinomas (4/16 vs 10/14) in groups receiving 0.01% and 0.025% BBN, respectively, compared to controls ($p < 0.02$) (Miyata *et al.*, 1978).

4.2.1.4 Skin (Table 23)

(a) Mouse

Four groups of 20 or 10 female Swiss mice, 8 weeks of age, were treated as follows: (1) MCA was applied on the shaved skin (0.1 mL/mouse as a 0.3% solution in acetone) twice weekly for the first five weeks and then once weekly during the sixth to ninth weeks (14 applications); (2) MCA as in group 1 and, during the third to fifth weeks, retinyl palmitate (6 mg/0.1 mL acetone/mouse) was applied to the same treated areas of the skin twice weekly (6 applications) and subsequently, once weekly during the sixth to ninth weeks (4 applications); (3) retinyl palmitate alone as group 2 (10 applications) and (4) acetone alone (14 applications). From the 10th week on, no further treatment was given until the end of the experiment at 23 weeks. Groups 1 and 2 comprised 20 mice per group and groups 3 and 4, 10 mice per group. The tumours were classified histopathologically. In the group treated with MCA and retinyl palmitate, the incidence of mice with papillomas (3/20) was lower than in mice treated with

Table 23. Chemopreventive activity of vitamin A and retinyl esters on skin carcinogenesis

Species, strain, sex	No. of animals/group	Carcinogen, dose, route and duration of administration	Vitamin A (dose)	Treatment relative to carcinogen	Preventive efficacy	Reference
Mouse, Swiss, female	20/group	MCA, 0.1 ml of 0.3% solution in acetone, applied to the shaved skin (14 applications)	Retinyl palmitate, 6 mg/0.1 mL in acetone applied on the skin	During	Effective (papillomas) Ineffective (carcinomas)	Abdel-Galil *et al.* (1984)
Mouse, Skh-hr1, Hairless, female 10–12 weeks old	20/group	UVR; 280–700 nm, 5 days/week for 12 weeks	Retinyl palmitate 60 IU or 300 IU, 3 times per week by stomach tube until death	Before and during	Ineffective	Kelly *et al.* (1989)
Mouse, CD-1, female	35/group	DMBA, single topical application of 150 nmol; promoted twice weekly with 8 nmol TPA for 21 weeks	Retinyl palmitate, 60, 200, 700/350 IU per g diet	During promotion with TPA	Effective (papillomas)	Gensler *et al.* (1987)
Mouse, C3H/HeN, female, 6 weeks old	20–30/group	UVB radiation, 280–320 nm, 30 min/day, 5 days/week from week 18–42	Retinyl palmitate, 0, 120 IU per g diet for 45.5 weeks	Before and during	Inconclusive	Gensler *et al.* (1990)
Mouse, Oslo/Bom inbred hairless, female (age not given)	44/group	UVB (280–320 nm) or UVAB (280–380 nm) for 18 weeks	0.3–0.6 mg retinol/kg diet or 4–6 mg/kg diet for 78 weeks	1 month before and during	Increased tumour incidence	Mikkelsen *et al.* (1998)

Abbreviations: DMBA, 7,12-dimethylbenz[*a*]anthracene; MCA, 3-methylcholanthrene; TPA, 12-*O*-tetradecanoylphorbol 13-acetate

MCA only (9/19). The incidence of mice with carcinomas was similar in both groups (4/20 versus 5/19). No skin tumour was seen in control groups (Abdel-Galil *et al.*, 1984).

Four groups of 35 female CD-1 mice, 6 weeks of age, were treated with a single topical application of 150 nmol of DMBA in 0.2 mL acetone and, starting three weeks later, 12-*O*-tetradecanoylphorbol 13-acetate (TPA) (8 nmol) was applied in 0.2 mL acetone twice weekly for 21 weeks. The control diet (AIN-76A) contained 3.9 IU retinyl palmitate/g diet. Mice were fed diets supplemented with retinyl palmitate for 21 weeks at a level of 60, 200 or 700 IU/g diet (the high dose was reduced to 350 IU per g of diet after five weeks). There were 14.3 ± 2.6, 8.2 ± 2.0 and 3.4 ± 1.2 papillomas per mouse in the groups on the low, medium and high doses of retinyl palmitate, respectively, versus 15.6 ± 2.7 in controls, corresponding to reductions of 9%, 37% ($p < 0.02$) and 65% ($p < 0.02$) (Gensler *et al.*, 1987).

Groups of 20 inbred, hairless (Skh-hr1) female mice, 10–12 weeks of age, were exposed to 280–700 nm ultraviolet light on five days per week for 12 weeks. The initial daily exposure was 0.53 J/cm^2. Subsequent exposure times were increased by 20% every two weeks. After 12 weeks, the exposure was increased to 1.60 J/cm^2 and from that time up to 25 weeks following the start of treatment was held at that level at a frequency of twice weekly. Retinyl palmitate (60 or 30 IU) was dissolved in arachis oil and administered intragastrically three times weekly beginning two weeks before the start of treatment and continuing until death. All tumours were classified histopathologically. The incidence of skin tumours (papillomas and carcinomas) in both retinyl palmitate-treated groups was similar to that in controls (Kelly *et al.*, 1989).

Groups of 20–30 female C3H/HeNCrlBR mice, six weeks of age, were fed: (1) an AIN-76A basal diet containing 4 IU retinyl palmitate/g diet; (2) basal diet supplemented with 120 IU retinyl palmitate/g diet; (3) basal diet supplemented with 1% canthaxanthin or (4) basal diet supplemented with 1% canthaxanthin plus 120 IU retinyl palmitate/g diet. The animals were maintained on their respective diets for at least 45 1/2 weeks. During weeks 18–42, the shaved dorsal skin of the animals was irradiated with ultraviolet light (280–320 nm) for 30 minutes per day on five days per week. There was no significant difference in tumour incidence in irradiated mice fed retinyl palmitate (32%), canthaxanthin (36%), retinyl palmitate plus canthaxanthin (31%) and controls (37%). Dietary supplementation with retinyl palmitate, however, significantly reduced the mean skin tumour burden at four weeks (sum of the tumour areas on each mouse) by 41% in comparison with controls ($p < 0.008$; analysis of variance of the log tumour burden). Canthaxanthin reduced the tumour burden per mouse by 45%, whereas canthaxanthin plus retinyl palmitate treatment resulted in a 68% reduction in tumour burden, indicating no interaction between retinyl palmitate and canthaxanthin (Gensler *et al.*, 1990). [The Working Group noted that the measurement of skin tumour burden was rather unusual for skin carcinogenesis studies.]

Four groups of 44 hairless female mice of the Oslo/Bom inbred strain [age not specified] were fed a standard laboratory diet containing low (0.3–0.6 mg/kg diet) or high (4–6 mg/kg diet) levels of retinol. After the mice had been fed these diets for one month, the animals were exposed daily to irradiation with ultra-violet B (280–320 nm) or B and A (280–380 nm) for 18 weeks followed by an observation period of up to 60 weeks. After one year, the incidence of skin squamous cell carcinomas was 49–63% in the high-retinol group versus 25–39% in the low-retinol group ($p < 0.003$). Two months later, these figures were 66–72% versus 50–53% for the high- and low-retinol groups, respectively ($p = 0.01$) (Mikkelsen *et al.*, 1998).

4.2.1.5 Oesophagus and forestomach (Table 24)

(a) Mouse

Three groups of 150 male ICR/Jcl mice, four weeks of age, were treated as follows: (1) no treatment with carcinogen; (2) 0.2 mg BP in 0.2 mL corn oil by gavage twice a week to a total dose of 2.0 mg and (3) 2.0 mg BP in 0.2 mL corn oil to a total dose of 20.0 mg. Each of these groups was divided into five subgroups

Table 24. Chemopreventive effects of vitamin A and retinyl esters in other organs

Species, strain, sex	No. of animals	Carcinogen (dose/route)	Vitamin A (dose/route)	Treatment relative to carcinogen	Preventive efficacy	Reference
Forestomach						
Mouse, ICR/Jcl, male, 4 weeks old	30/group	BP in corn oil by gavage; twice weekly to total dose of 2 mg or 20 mg	Retinyl palmitate: 5 IU, 50 IU or 200 IU/day in diet for 66 weeks	Before and during in one group and after in another group	Effective at low dose of BP on incidence of papillomas Ineffective at high dose of BP	Yamada *et al.* (1995)
Oesophagus						
Rat, Sprague-Dawley, sex and age unspecified	60/group	*N*-Nitroso-*N*-methyl-benzylamine, 2.5 mg/kg bw twice a week for 5 weeks, intragastrically	Retinyl acetate: 0.3 mg/kg, 2.2 mg/kg or 29.9 mg/kg in diet for 15 weeks	Before, during and after carcinogen	Ineffective	Nauss *et al.* (1987)
Large intestine						
Rat, Sprague-Dawley, male, age not given	50/group	Aflatoxin B_1 in semi-synthetic diet (0.1 mg/kg diet)	Retinyl palmitate: 5, 50 or 500 mg/day/rat in diet for 24 months	During	Colon carcinomas developed in the retinyl palmitate deficient group	Newberne & Rogers (1973)
Rat, Sprague-Dawley, male, weanling	12/group	1,2-Dimethylhydrazine, two injections 25 mg/kg bw three days apart	Retinyl palmitate: 200 IU/g diet for 12 weeks	After		Cassand *et al.* (1997)
Rat, Fischer 344, male, 6 weeks old	9 or 10/group	Azoxymethane, two intraperitoneal injections 15 mg/kg bw one week apart	Retinyl palmitate: 0.5 or 1.0 mmol/kg diet for 5 weeks	After		Zheng *et al.* (1997)
Thyroid						
Rat, Fischer 344, male	15/group	*N*-Nitroso-*N*-bis(2-hydroxy-propyl)amine, 2800 mg/kg bw, one subcutaneous injection + 0.2% thiourea in drinking-water	Retinyl acetate: 320 IU/g diet	After	Ineffective	Mitsumori *et al.* (1996)

Abbreviations: BP, benzo[*a*]pyrene

of 30 mice each, which were given dietary retinyl palmitate supplementations as follows: (A) 50 IU/day (assumed to be an adequate vitamin A intake for ICR/Jcl mice); (B) 5 IU/day; (C) 200 IU/day (retinyl palmitate supplementation was given during the initiation and post-initiation phases); (D) 5 IU/day, and (E) 200 IU/day. Groups D and E both received 50 IU retinyl palmitate/day during the initiation phase and the low (5 IU) and high (200 IU) levels during the post-initiation phase. All surviving animals were killed at 66 weeks. In animals treated with the low dose of BP, the incidence of forestomach papilloma-bearing mice of the two high retinyl palmitate groups was significantly lower ($p < 0.05$) in comparison with the group given 50 IU/day (2/27, 2/27 versus 8/28), whereas the incidence of forestomach papilloma-bearing mice in the group given 5 IU retinyl palmitate/day was significantly ($p < 0.05$) higher than in controls fed 50 IU/day (15/26 vs 8/28). Carcinomas were not induced with this low dose of BP. No effect of retinyl palmitate was seen on papillomas and carcinomas in the groups treated with a high dose of BP (Yamada *et al.*, 1995).

(b) Rat

Groups of 65 Sprague–Dawley rats (sex unspecified) were fed semipurified diets containing levels of retinyl acetate which were either adequate (2.2 mg/kg of diet), deficient (0.30 mg/kg of diet) or high (29.9 mg/kg of diet). After four weeks of adaptation to the experimental diets, 60 rats per dietary group received 2.5 mg/kg bw *N*-nitroso-*N*-methylbenzylamine (NMBA), twice a week for five weeks. NMBA was dissolved in 10% ethanol and administered by gavage. Five animals in each group received vehicle only. Fifteen weeks after the last dose of carcinogen, the animals were killed and the oesophagus was evaluated for presence of neoplasms, using routine histopathological procedures. The incidence and multiplicity of carcinomas, papillomas and preneoplastic lesions were similar in all groups, indicating that dietary retinyl acetate levels did not influence oesophageal tumour development induced by NMBA (Nauss *et al.*, 1987).

4.2.1.6 Large intestine (Table 24)

(a) Rat

Groups of 50 male Sprague–Dawley rats were maintained on a semi-synthetic diet containing aflatoxin B_1 at a concentration of 0.1 mg/kg diet. Retinyl palmitate was mixed into the diet to provide 5, 50 or 500 mg/day per rat based on measured food consumption. The study was carried out for 24 months. Six colon carcinomas developed in the retinyl palmitate-deficient group. No such tumours were found in any of the other groups (Newberne & Rogers, 1973).

Sprague–Dawley weanling male rats were divided into two groups of 12 animals. Rats were treated with 50 mg/kg 1,2-dimethylhydrazine hydrochloride (DMH) in two injections of 25 mg/kg three days apart. Starting one week after the last injection, rats received either semi-synthetic diet as control or diet supplemented with 200 IU/g retinyl acetate for 12 weeks. Animals were then killed and the colon was fixed and stained for aberrant crypts, considered to be precursors of colon cancers. Adenocarcinomas were also removed and microscopically identified. Retinyl acetate-treated animals exhibited 4.2 aberrant crypts per cm^2, compared with 5.7 per cm^2 in controls. The total number of adenocarcinomas per rat decreased from 364 to 252 ($p < 0.08$) (Cassand *et al.*, 1997).

Two groups of 9 or 10 male Fischer 344 rats, six weeks of age, were treated with retinyl palmitate at concentrations of 0.5 or 1.0 mmol/kg in AIN-76A basal diet (263 or 525 ppm) for five weeks after prior treatment with two weekly intraperitoneal injections of 15 mg/kg bw azoxymethane for two weeks. The numbers of preneoplastic aberrant crypt foci per rat were 51.3 $\pm$ 5.38 and 36.2 $\pm$ 6.49 at the low and high doses of retinyl acetate, respectively, whereas the control group had 65.2 $\pm$ 5.22 aberrant crypt foci per rat; this constitutes a 44.5% reduction at the highest dose ($p < 0.01$) (Zheng *et al.*, 1997).

4.2.1.7 Liver

(a) Rat

In the study of Newberne and Rogers (1973) described in Section 4.2.1.6, the number of aflatoxin B_1-induced liver tumours was unaffected by retinyl palmitate administration.

4.2.1.8 Thyroid (Table 24)

(a) Rat

Four groups of male Fischer 344 rats, four weeks of age, received a single subcutaneous injection of 2800 mg/kg bw *N*-nitroso-*N*-bis(2-hydroxypropyl)amine (DHPN). Group 1 (5 rats), received tap water; group 2 (5 rats) received 0.1% retinyl acetate in the diet; group 3 (15 rats) received 0.2% thiourea; and group 4 (15 rats) received thiourea plus retinyl acetate in the diet. Animals were killed 20 weeks after the carcinogen treatment. No thyroid tumours were found in animals receiving DHPN and either retinyl acetate alone or basal diet. In animals treated with DHPN plus thiourea, the incidence of follicular-cell tumours [exact histology not given] was similar to that in the group given DHPN + thiourea + retinyl acetate (Mitsumori *et al.*, 1996).

4.2.1.9 Other sites

(a) Mouse

Groups of 20 male CBA/J mice, six weeks of age, were fed basal diet (Teklad) for three days before experiment. They were then fed either control diet or diet supplemented with 150 mg/kg of diet retinyl palmitate throughout the experiment. All animals were inoculated with murine sarcoma virus at three concentrations. Animals were killed 20 days after virus inoculation. At a 1×10^{-3} dilution of virus (low dose), the tumour incidence [site not specified] was 37.5% in controls compared with 0% in the retinyl palmitate-treated group. At higher virus concentrations (1×10^{-2} and 1×10^{-1}), there was a 62.5% tumour incidence in controls compared with 25% and 12.5% incidence, respectively, in the retinyl palmitate-treated groups. Results showed significant suppression of virally induced sarcoma by retinyl palmitate ($p < 0.01$, *t*-test) (Seifter *et al.*, 1983).

Pregnant female albino rats [number unspecified] were given 75 mg/kg bw *N*-ethyl-*N*-nitrosourea by intravenous injection. The mothers were treated with retinyl acetate (300 IU/g diet) during the suckling phase of the offspring. The pups were given retinyl acetate in diet. Animals were allowed to live until natural death. Tumours of the nervous system and kidney developed in all offspring. Retinyl acetate did not affect the incidence of any tumour type or the average life span of the rats (Alexandrov *et al.*, 1991).

4.2.2 Intermediate biomarkers

The ability of retinol, retinal, retinoic acid and retinyl esters to modulate intermediate biomarkers was evaluated in rodents (mouse, rat, hamster) treated with a variety of genotoxic agents, including alkylating agents, polycyclic aromatic hydrocarbons, aromatic amines, mycotoxins and cytostatic agents. The investigated end-points included DNA or RNA binding, DNA damage and clastogenic damage, as assessed by determining the frequency of sister chromatid exchanges, micronuclei and chromosomal aberrations. Table 25 summarizes the outline of these studies and the results obtained.

Five studies evaluated the formation of DNA or RNA adducts under various experimental conditions. The ability of ^{14}C-labelled 2-acetylaminofluorene to bind DNA or RNA of liver cells was not affected in Sprague–Dawley rats given retinyl palmitate orally twice a week according to a dose escalation schedule from the 1st to the 12th week before intraperitoneal injection of the carcinogen (Rondahl *et al.*, 1985). The formation of DNA adducts was investigated by ^{32}P-postlabelling analysis in skin cells of CD–1 mice receiving topical applications of (7*S*,8*S*)-dihydroxy-7,8-dihydrobenzo[*a*]pyrene [(+)-BP-7,8-diol] and of TPA. TPA was administered both 24 h earlier and simultaneously with (+)-BP-7,8-diol. The topical application of retinoic acid had no effect on the formation of (–)-*syn*-BP diol epoxide adducts, whereas it significantly decreased the formation of (–)-*anti*-isomer adducts, but only when co-administered with the second TPA application (Marnett & Ji, 1994). Retinyl acetate, given by gavage for seven days, attenuated the binding of [^{3}H]benzo[*a*]pyrene, given as a single intraperitoneal injection, to the DNA of hepatocytes and stomach cells, but did not affect its binding to the DNA of lung and kidney cells (McCarthy *et al.*, 1987). Administration of retinyl acetate with the diet for two weeks decreased the formation of ^{32}P-postlabelled DNA adducts in mammary cells of Sprague–Dawley rats receiving a single treatment by gavage with 7,12-dimethylbenz[*a*]anthracene (DMBA)

Table 25. Assay of retinol, retinal, retinoic acid and retinyl esters for ability to modulate intermediate biomarkers in animal models

End-point	Code[a]	Modulator (administration schedule and tested doses)[b]	Genotoxic agent (administration schedule and tested doses)[b]	Animal strains and species, and cells	Investigated effect	Result[c]	Inhibitory dose (IDx)[d]	Reference
D	BVP	Retinyl palmitate p.o. twice a week for 12 weeks (0.9 to 7.2 mg/administration)	2-Acetylaminofluorene, single i.p. injection (0.94 mg/kg bw)	Sprague–Dawley rats, liver cells	Binding to RNA	–	NA	Rondahl *et al.* (1985)
D	BVD	Retinyl palmitate p.o. twice a week for 12 weeks (0.9 to 7.2 mg/administration)	2-Acetylaminofluorene, single i.p. injection (0.94 mg/kg bw)	Sprague–Dawley rats, liver cells	Binding to DNA	–	NA	Rondahl *et al.* (1985)
D	BVD	Retinyl acetate by gavage for 7 days (80 mg/kg bw)	[^{3}H]BP, single i.p. injection (2 mg/kg bw)	Sprague–Dawley rats, hepatocytes	Binding to DNA	(+)	80 mg/kg bw (ID27)	McCarthy *et al.* (1987)
D	BVD	Retinyl acetate by gavage for 7 days (80 mg/kg bw)	[^{3}H]BP, single i.p. injection (2 mg/kg bw)	Sprague–Dawley rats, stomach cells	Binding to DNA	(+)	80 mg/kg bw (ID32)	McCarthy *et al.* (1987)
D	BVD	Retinyl acetate by gavage for 7 days(80 mg/kg bw)	[^{3}H]BP, single i.p. injection (2 mg/kg bw)	Sprague–Dawley rats, lung cells	Binding to DNA	–	NA	McCarthy *et al.* (1987)
D	BVD	Retinyl acetate by gavage for 7 days (80 mg/kg bw)	[^{3}H]BP, single i.p. injection (2 mg/kg bw)	Sprague–Dawley rats, kidney cells	Binding to DNA	–	NA	McCarthy *et al.* (1987)
D	BVD	Retinoic acid (50 µg topical)	(7S,8S)-Dihydroxy-7,8-dihydrobenzo[*a*]pyrene, topical (200 nmol) + TPA, topical (10 nmol)	CD-1 mice, skin cells	Binding to DNA [(–) *anti*-BPDE-dG adducts]	+	50 µg (ID50)	Marnett & Ji (1994)
D	BVD	Retinoic acid (50 µg topical)	(7S,8S)-Dihydroxy-7,8-dihydrobenzo[*a*]pyrene, topical (200 nmol) + TPA, topical (10 nmol)	CD-1 mice, skin cells	Binding to DNA [(+)*syn*-BPDE-dG adducts]	–	NA	Marnett & Ji (1994)
D	BVD	Retinyl acetate p.o. for 2 weeks (328 mg/kg diet)	DMBA, single gavage administration (25 mg/kg bw)	Sprague–Dawley rats, mammary cells	Binding to DNA	(+)	328 mg/kg diet (ID30)	Amagase *et al.* (1996)

Table 25 (contd)

End-point	Code[a]	Modulator (administration schedule and tested doses)[b]	Genotoxic agent (administration schedule and tested doses)[b]	Animal strains and species, and cells	Investigated effect	Result[c]	Inhibitory dose (IDx)[d]	Reference
D	BVD	Retinol by gavage for 7 days (100 mg/kg bw/day)	Ochratoxin A single gavage administration (2 mg/kg bw)	Swiss mice, kidney cells	Binding to DNA	+	100 mg/kg bw (ID59)	Grosse *et al.* (1997)
D	DVA	Retinyl palmitate p.o. for 8 weeks (5–500 IU/g diet)	Aflatoxin B_1, single i.p. injection (1 mg/kg bw)	Sprague–Dawley rats, hepatocytes	Single-strand breaks	(+)	5 IU/g diet (ID65)	Decoudu *et al.* (1992)
D	DVA	Vitamin A [unspecified] p.o. from day 7 of pregnancy to day 10 post delivery (90 IU)	DMBA, single dose by gavage in the female offspring (80 mg/kg bw), and phenobarbital, single dose by gavage 24 hours earlier (60 mg/kg bw)	Sprague–Dawley rats, liver cells	Single-strand breaks	+	90 IU (ID66)	Bolognesi *et al.* (1992)
D	DVA	Vitamin A [unspecified] p.o. from day 7 of pregnancy to day 10 post delivery (90 IU)	DMBA, single dose by gavage in the female offspring (80 mg/kg bw)	Sprague–Dawley rats, liver cells	Single-strand breaks	(+)	90 IU (ID39)	Bolognesi *et al.* (1992)
D	DVA	Vitamin A [unspecified] p.o. from day 7 of pregnancy to day 10 post delivery (90 IU)	DMBA , single dose by gavage in the female offspring (80 mg/kg bw), and phenobarbital, single dose by gavage 24 hours earlier (60 mg/kg bw)	Sprague–Dawley rats, mammary gland cells	Single-strand breaks	+	90 IU (ID54)	Bolognesi *et al.* (1992)
D	DVA	Vitamin A [unspecified] p.o. from day 7 of pregnancy to day 10 post delivery (90 IU)	DMBA, single dose by gavage in the female offspring (80 mg/kg bw)	Sprague–Dawley rats, mammary gland cells	Single-strand breaks	+	90 IU (ID50)	Bolognesi *et al.* (1992)
D	DVA	Vitamin A [unspecified] p.o. from day 7 of pregnancy to day 10 post delivery (90 IU)	MNU, single dose by gavage in the female offspring (50 mg/kg bw), and phenobarbital, single dose by gavage 24 hours earlier (60 mg/kg bw)	Sprague–Dawley rats, liver cells	Single-strand breaks	–	NA	Bolognesi *et al.* (1992)

Table 25 (contd)

End-point	Code[a]	Modulator (administration schedule and tested doses)[b]	Genotoxic agent (administration schedule and tested doses)[b]	Animal strains and species, and cells	Investigated effect	Result[c]	Inhibitory dose (IDx)[d]	Reference
D	DVA	Vitamin A [unspecified] p.o. from day 7 of pregnancy to day 10 post delivery (90 IU)	MNU single dose by gavage in the female offspring (50 mg/kg bw), and phenobarbital, single dose by gavage 24 hours earlier (60 mg/kg bw)	Sprague–Dawley rats, mammary gland cells	Single-strand breaks	–	NA	Bolognesi *et al.* (1992)
D	DVA	Retinyl palmitate p.o. for 2 weeks after 9 weeks on a vitamin A deficient diet (50-100 IU/day)	Aflatoxin B_1, single i.p. injection (7 mg/kg bw)	Wistar rats, hepatocytes	Single-strand breaks	(+)	100 IU/day (ID15)	Webster *et al.* (1996)
D	DVA	Retinyl palmitate p.o. for 2 weeks (50–100 IU/day)	*N*-Nitrosodimethylamine single i.p. injection (10 mg/kg bw)	Wistar rats, hepatocytes	Single-strand breaks	(+)	100 IU/day (ID20)	Webster *et al.* (1996)
S	SVA	Retinyl acetate p.o. for 10 weeks (20 mg/kg diet)	Aflatoxin B_1 single s.c. injection (16–32 mg/kg bw)	C57BL/6J mice, bone marrow cells	Sister chromatid exchanges	+	20 mg/kg diet (ID70)	Qin & Huang (1986)
S	SVA	Retinyl acetate p.o. for 10 weeks (20 mg/kg diet)	BP single i.p. injection (200–400 mg/kg bw)	C57BL/6J mice, bone marrow cells	Sister chromatid exchanges	-	NA	Qin & Huang (1986)
S'	SVA	Retinyl acetate p.o. for 10 weeks (20 mg/kg diet)	Cyclophosphamide single i.p. injection (8–16 mg/kg bw)	C57BL/6J mice, bone marrow cells	Sister chromatid exchanges	–	NA	Qin & Huang (1986)
M	MVM	Retinyl palmitate by gavage, twice a week for 7 weeks (32 mg/kg bw)	Cyclophosphamide single i.p. injection (10–50 mg/kg bw)	NMRI mice, bone marrow cells	Micronuclei	–	NA	Busk *et al.* (1984)
M	MVM	Vitamin A [unspecified], single oral administration (150 IU)	BP, single oral administration (75 mg/kg bw) 1 hour before vitamin A	Swiss albino mice, bone marrow cells	Micronuclei	+	150 IU (ID100)	Rao *et al.* (1986)
M	MVM	Retinyl palmitate by gavage for 14 weeks (132 IU/kg bw/day)	Aflatoxin B_1 by gavage for 14 weeks (0.05 µg/kg bw/day)	Swiss albino mice, erythrocytes from bone marrow	Micronuclei	+	132 IU/kg bw/day (ID66)	Sinha & Kumari (1994)

Table 25 (contd)

End-point	Code[a]	Modulator (administration schedule and tested doses)[b]	Genotoxic agent (administration schedule and tested doses)[b]	Animal strains and species, and cells	Investigated effect	Result[c]	Inhibitory dose (IDx)[d]	Reference
M	MVR	Retinyl palmitate p.o. for 5 weeks (20 000 U/kg diet)	4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone, single i.p. injection (25–100 mg/kg bw), followed by partial hepatectomy	Fischer rats, hepatocytes	Micronuclei	+	20,000 U/kg diet (ID95)	Alaoui–Jamali *et al.* (1991b)
C	CBA	Retinol, single gavage administration (1–25 mg/kg bw)	Busulfan (myleran), single gavage administration (50 mg/kg bw)	Chinese hamsters, bone marrow cells	Chromosomal aberrations	–	NA	Renner (1985)
C	CBA	Retinol, single gavage administration (1–25 mg/kg bw)	Cyclophosphamide, single gavage administration (20 mg/kg bw)	Chinese hamsters, bone marrow cells	Chromosomal aberrations	–	NA	Renner (1985)
C	CBA	Retinol, single gavage administration (1–25 mg/kg bw)	Thio-TEPA, single gavage administration (10 mg/kg bw)	Chinese hamsters, bone marrow cells	Chromosomal aberrations	–	NA	Renner (1985)
C	CBA	Retinol, single gavage administration (1–25 mg/kg bw)	Methyl methanesulfonate, single gavage administration (40 mg/kg bw)	Chinese hamsters, bone marrow cells	Chromosomal aberrations	–	NA	Renner (1985)
C	CBA	Retinyl palmitate by gavage for 14 weeks (132 IU/kg bw/day)	Aflatoxin B_1 by gavage for 14 weeks (0.05 µg/kg bw/day)	Swiss albino mice, bone marrow cells	Chromosomal aberrations	+	132 IU/kg bw/day (ID73)	Sinha & Kumari (1994)
C	CBA	Retinyl palmitate by gavage for 14 days (132 IU/kg bw/day)	Ochratoxin by gavage for 14 days (1 µg/kg bw)	Swiss albino mice, bone marrow cells	Chromosomal aberrations	+	132 IU/kg bw/day (ID68)	Kumari & Sinha (1994)
C	CCC	Retinyl palmitate by gavage for 14 weeks (132 IU/kg bw/day)	Aflatoxin B_1 by gavage for 14 weeks (0.05 µg/kg bw/day)	Swiss albino mice, primary spermatocytes	Chromosomal aberrations	+	132 IU/kg bw/day (ID85)	Sinha & Kumari (1994)
C	CCC	Retinyl palmitate by gavage for 14 days (132 IU/kg bw/day)	Ochratoxin by gavage for 14 days (1 µg/kg bw)	Swiss albino mice, primary spermatocytes	Chromosomal aberrations	+	132 IU/kg bw/day (ID67)	Kumari & Sinha (1994)

[a] See Appendix 2.

[b] Doses of compounds are as reported by the authors

[c] +, inhibition of the investigated end-point *(≥ID50) ; (+), weak inhibition of the investigated end-point ≤ID50); –, no inhibition of the investigated end-point*

[d] IDx, dose inhibiting the x % of the investigated end-point; NA, not applicable.

Abbreviations: BP, benzo[*a*]pyrene; BPDE, benzo[*a*]pyrene diol epoxide; DMBA, 7,12-dimethylbenz[*a*]anthracene; MNU, *N*-methylnitrosourea; TPA, 12-*O*-tetradecanoylphorbol 13-acetate

24 h before killing. The levels of DNA adducts, most of which were identified as the *anti*-dG adduct, were significantly decreased in rats receiving the retinyl acetate-supplemented diet compared with rats fed the basal diet. Moreover, a further significant decrease in DNA adducts was observed following combined treatment with retinyl acetate and either garlic (20 g/kg diet) or garlic plus selenite (0.5 mg/kg diet) (Amagase *et al.*, 1996). A single administration by gavage of the mycotoxin ochratoxin resulted in the formation of 13 distinct DNA adducts in kidney cells of Swiss mice, as detected by ^{32}P-postlabelling. Pretreatment of mice with retinol by gavage for seven days caused a significant decrease in total adduct levels. In particular, seven adducts were markedly decreased and five adducts were no longer detectable, the major adduct being the only one which was unaffected in retinol-pretreated mice (Grosse *et al.*, 1997).

In a rat liver bioassay with initiation by *N*-nitrosodiethylamine (DEN) and promotion by polybrominated biphenyls, feeding 200 IU retinyl acetate/g of diet from day 7 to day 180 (end of study) decreased the number and volume of γ-glutamyltranspeptidase-positive foci, compared with the group given a low dose (2 IU/g of diet). Only the difference in percentage of volume occupied by foci between the high- and low-dose groups was significant (Rezabek *et al.*, 1989).

In a rat liver assay for foci of hepatocellular alterations with DEN initiation and 2-acetylaminofluorene promotion, retinyl acetate given as an intragastric dose of 10 mg/kg bw every other day throughout the study did not decrease the number and mean size of γ-glutamyltranspeptidase-positive foci compared with controls (Moreno *et al.*, 1995).

Two groups of 20 male weanling albino Wistar rats (Cpb/WU) were given intraperitoneal injections of 30 mg/kg bw azaserine at 19 days of age. Twelve days after initiation, the animals were fed a semisynthetic diet (AIN-based) high in saturated fat (20% lard) without (controls) or with 100 IU of retinyl acetate and retinyl palmitate (50:50 ratio). Four months after initiation, all animals were killed and the number and size of acidophilic foci were determined. The area as % of pancreas occupied by acidophilic focus tissue was significantly lower ($p < 0.05$) in the group maintained on the diet supplemented with retinyl acetate/retinyl palmitate (Woutersen & van-Garderen-Hoetmer, 1988).

Administration of retinyl palmitate in the diet significantly attenuated the induction of DNA single-strand breaks, as assessed by alkaline elution assay, in hepatocytes of Sprague-Dawley rats receiving a single intraperitoneal injection of aflatoxin B_1 (Decoudu *et al.*, 1992) and in hepatocytes of Wistar rats receiving single intraperitoneal injections of aflatoxin B_1 and *N*-nitrosodimethylamine (Webster *et al.*, 1996). Using the same technique, vitamin A [unspecified], given to Sprague-Dawley rats during embryonal and fetal life, gave protection against the induction of DNA single-strand breaks in liver and mammary gland cells of the female progeny treated by gavage with a single dose of DMBA. However, no protective effect was observed in rats treated with MNU (Bolognesi *et al.*, 1992).

Qin and Huang (1986) compared the ability of retinyl acetate, given in the diet for 10 weeks, to decrease the frequency of sister chromatid exchanges in bone marrow cells of C57BL/6J mice treated with either aflatoxin B_1, BP or cyclophosphamide by intraperitoneal injection. Administration of retinyl acetate yielded a concentration of 274 µg vitamin A per g liver, versus 38 µg/g in control mice. A protective effect was only observed towards aflatoxin B_1 (Busk *et al.*, 1984). A lack of modulation of sister chromatid exchanges induced by cyclophosphamide was also observed in bone marrow cells of NMRI mice receiving retinyl palmitate by gavage, twice a week for seven weeks, before the intraperitoneal injection of this cytostatic drug (Busk *et al.*, 1984). Conversely, oral retinyl palmitate produced a significant decrease in micronuclei in peripheral blood erythrocytes of Swiss albino mice co-treated by gavage with aflatoxin B_1 (Sinha & Kumari, 1994) in bone marrow cells of Swiss albino mice receiving BP orally (Rao *et al.*, 1986), and in hepatocytes of Fischer rats receiving an intraperitoneal injection of 4-(*N*-nitrosomethylamino)-1-(3-pyridyl)-1-butanone

(NNK), followed by partial hepatectomy (Alaoui-Jamali *et al.*, 1991b).

In Chinese hamsters, a single administration of retinol by gavage did not significantly affect the increased frequency of chromosomal aberrations induced in bone marrow cells either by the simultaneous administration by gavage of busulfan or cyclophosphamide, or administration by gavage of aziridine 1,1',1''-phosphinothioylidynetris (thiotepa) 2 h later, or intraperitoneal injection of methyl methanesulfonate 6 h later (Renner, 1985). Retinyl palmitate decreased the induction of chromosomal aberrations in bone marrow cells and spermatocytes of Swiss albino mice co-treated by gavage with either ochratoxin for 14 days (Kumari & Sinha, 1994) or aflatoxin B_1 for 14 weeks (Sinha & Kumari, 1994). In both studies, retinyl palmitate also decreased the frequency of spermatozoa showing abnormal head morphology due to treatment with these mycotoxins.

4.2.3 In-vitro models

4.2.3.1 Modulation of cell proliferation and differentiation

Most studies of cell proliferation and differentiation have been carried out with malignant cells and few with normal cells or immortalized cells. None of these cell systems is an optimal representative of premalignant cells. Therefore, the findings cannot be related directly to cellular processes that are modulated during carcinogenesis or during chemoprevention. Nonetheless, the findings point to possible mechanisms that might mediate effects on chemoprevention *in vivo*.

Vitamin A (retinol) can be converted to several classes of active metabolites: retinoic acids (all-*trans*-retinoic acid, that can be isomerized to 9-*cis*-retinoic acid and 13-*cis*-retinoic acid), 4-oxoretinol, and two retro-retinoids, anhydroretinol and 14-hydroxy-4,14-retro-retinol (14-HRR). All of these metabolites exert distinct effects on the growth, differentiation and apoptosis of various normal and malignant cells in culture.

Lotan and Nicolson (1977) described the ability of retinyl acetate and retinoic acid, both at 10 µmol/L, to inhibit the proliferation of a large number types of untransformed, transformed and malignant rodent and human cells in culture. The susceptible cell lines represented many different histological tumour types such as neuroblastoma, sarcoma, melanoma, mammary carcinoma, lymphoma, myeloma and lymphosarcoma. The sensitivity to the retinoids was independent of the type of transforming agent used to derive some of the rodent cell lines, including chemical carcinogens such as BP, MCA, DMBA, mineral oil, polyoma virus, Abelson leukaemia virus, or spontaneous transformation. Retinyl acetate was usually less potent than retinoic acid. Subsequent studies with selected cell lines demonstrated that the growth-inhibitory effects were dose- and time-dependent and were reversible upon removal of the retinoid from the medium (Lotan *et al.*, 1978). Because initial studies demonstrated that retinyl acetate was less potent in inhibition of cell growth than retinoic acid, most subsequent studies have employed the acid metabolite. However, this section describes only studies with retinol and retinyl esters, typically at pharmacological doses. The cells that have been analysed for response to retinol addition or removal from the growth medium include primary cultures (e.g., epidermal keratinocytes), immortalized cells (e.g., keratinocytes transfected with human papillomavirus E6 or SV40 T antigen) and malignant cells (e.g., established tumour cell lines). The type of effects that retinol exerted on the cells included modulation of cell proliferation and differentiation.

(a) Epidermal keratinocytes and squamous carcinoma cells

Exposure of primary cultures of mouse epidermal keratinocytes to retinyl acetate inhibits cell proliferation and suppresses squamous cell differentiation. The differentiation of cells treated with 40 µmol/L retinyl acetate was suppressed. Treated cells exhibited prolonged survival despite the inhibition of proliferation, due to a reduced rate of cell death. Such death normally follows squamous cell differentiation in culture (Yuspa *et al.*, 1977). Similarly, retinol inhibited calcium-induced stratification and terminal differentiation with keratinization in confluent

culture of cutaneous keratinocytes from the newborn rat. The addition of retinol to the medium enhanced features characteristic of the secretory epithelium, such as formation of an extensive endoplasmic reticulum, enlargement of the Golgi zone and an increase in the number of vacuoles. Thus, retinol redirected epithelial differentiation from a stratifying and keratinizing epithelium towards a secretory epithelium (Brown *et al.*, 1985).

Treatment of mouse epidermal keratinocytes with retinyl acetate affected several parameters presumed to be important in chemical carcinogenesis (Yuspa *et al.*, 1977). Whereas the activity of constitutive aryl hydrocarbon hydroxylase (AHH) was not significantly affected after exposure to retinyl acetate, the level of AHH induced by BP was reduced to 20% of that in controls. Further, in the presence of retinyl acetate, binding of DMBA to epidermal cell DNA was markedly decreased.

Retinyl acetate stimulated the outgrowth of human epidermal keratinocytes in primary skin cultures. Treated cultures exhibited higher mitotic index, higher labelling index and a larger growth fraction than control cultures. In addition, the number of keratohyaline granules in treated cultures decreased, indicating an effect on the differentiation process, although there was no evidence of mucous metaplasia (Chopra & Flaxman, 1975). The effects of retinol on the differentiation of various keratinocytes were defined in cellular and molecular terms by the studies of Fuchs and Green (1981) and Green and Watt (1982), who established that endogenous retinol present in the serum, when added to the growth medium, affects the differentiation of cultured keratinocytes derived from skin and from other stratified squamous epithelia. The removal of retinol from serum causes a reduction in cell motility, an increase in cell adhesiveness and inhibition of pattern formation. Further, removal of vitamin A leads to synthesis of a 67 kDa keratin characteristic of terminally differentiating epidermis and to much reduced synthesis of the 52 kDa and 40 kDa keratins typical of conjunctiva. The addition of retinyl acetate to the medium restored cell motility and pattern formation and enhanced the detachment of the most mature cells from the surface of the stratified epithelium. In addition, the production of the 67 kDa keratin was prevented and the synthesis of the 40 and 52 kDa keratins was stimulated. The formation of cross-linked envelopes, which occurs during the last stage of terminal differentiation, was inhibited by the addition of retinyl acetate.

Whereas the above studies used normal keratinocytes in short-term culture, a few studies have used immortalized keratinocytes as a model for premalignant cells *in vitro*. Two approaches have been successful for the immortalization technique, one using a recombinant retrovirus encoding the simian virus 40 (SV40) large T-antigen (Agarwal & Eckert 1990) and the other involving transfection of various normal human keratinocyte cell strains with human papillomavirus type 16 (HPV-16) DNA (Pirisi *et al.*, 1992). The SV40-T-immortalized human keratinocyte cells (cell line KER-1) do not form colonies in soft agar and are non-tumorigenic. The pattern of keratin gene expression in non-immortalized and KER-1 cells is similar, except that KER-1 cells express keratin 7 (K7), which is not expressed by non-immortalized keratinocytes. Incubation with retinol (0.2 or 2 μmol/L) results in a 40-fold increase in K7 expression in KER-1 cells. The formation of cross-linked cornified envelopes is reduced by retinol in both non-immortalized keratinocytes and KER-1 cells, without affecting the level of the envelope precursor, involucrin (Agarwal & Eckert 1990). Thus, the response of 'premalignant' keratinocytes to retinol is similar to that of normal cells in terms of modulation of differentiation.

Comparison of normal human foreskin keratinocytes (HKc) and four HPV16-immortalized HKc lines revealed that all the immortalized lines were 10- to 100-fold more sensitive than normal HKc to growth inhibition by retinol in both clonal and mass culture growth assays. In addition, the immortalized cell lines were more sensitive to modulation of keratin expression by retinol, despite a similar rate of uptake of [^{3}H]retinol by normal and immortalized cells. Retinoic acid, which can be formed from retinol in skin keratinocytes (Siegenthaler, 1990), also inhibited the growth

of immortalized cells preferentially and, further, was found to reduce the expression of the HPV-16 open reading frames of E2, E5, E6 and E7 two- to four-fold. These results suggest that the increased sensitivity of the immortalized cell lines to growth control by retinol and retinoic acid may be mediated by inhibition of the expression of HPV-16 oncogene products which are required for the maintenance of continuous growth (Pirisi *et al.*, 1992; Creek *et al.*, 1994). Retinoic acid treatment (1 nmol/L) of normal HKc, during or immediately following transfection with HPV-16 DNA, inhibited immortalization by about 95%. Overall, these results point to a possible biochemical basis for a role of dietary retinoids in chemoprevention of HPV-induced cancers (Creek *et al.*, 1994).

The antiproliferative and differentiation-suppressing effects of retinyl acetate have also been demonstrated in fully malignant epidermal keratinocyte cell lines (e.g., SCC-13), derived from human squamous cell carcinomas (Cline & Rice, 1983). The competence to form cross-linked envelopes in confluent SCC-13 cell cultures, which was about 50% in medium without retinyl acetate supplementation, was reduced to 10% after addition of retinyl acetate. A similar reduction was observed in the levels of involucrin. The results suggest that some potential differentiated character of malignant keratinocytes may be suppressed by vitamin A. During carcinogenesis, there is a loss of differentiation potential, which is apparent after the removal of vitamin A from the medium, as the maximal degree of differentiation attained by SCC cells is lower than that of normal keratinocytes (Kim *et al.*, 1984). Still, the SCC cells respond to changes in vitamin A levels in the medium, as shown by the altered expression of 67-kDa and 40-kDa keratins in SCCs from tongue and epidermis. When the vitamin A concentration in the medium was raised, the expression of the 40-kDa keratin increased. Conversely, a reduction in the amount of vitamin A led to increased expression of the 67-kDa keratin and the cells underwent stratification and terminal differentiation (Kim *et al.*, 1984).

(b) Normal and malignant airway epithelial cells

Extensive studies have been carried out with organ cultures and with normal bronchial epithelial cells derived from rat, rabbit, hamster, monkey and human trachea. These have demonstrated that vitamin A is required for the maintenance of proper differentiation *in vitro*. For example, the expression of a normal mucocilary epithelium in explants of rat trachea required supplementation with retinyl acetate. The explants secreted mucous glycoproteins into the medium and the production of the mucins was dependent upon the vitamin A status of the explant. In the absence of vitamin A (serum-free medium), the explants underwent a metaplastic change to a keratinizing squamous epithelium. The addition of 0.1, 2 or 10 µg retinyl acetate per mL of medium stimulated mucin synthesis within 24 hours which continued throughout the 21-day exposure period in a concentration-dependent fashion. The keratinizing squamous epithelium began to revert to a mucus-secreting tissue as early as 24 hours after addition of 10 µg retinyl acetate to the medium. The response was slower with the lower vitamin concentrations (Clark & Marchok 1979; Clark *et al.*, 1980). Studies with two carcinoma cell lines (T8 and 100 WT), derived from a mucus-secreting adenocarcinoma and a keratinizing squamous cell carcinoma, respectively, revealed that retinyl acetate added to medium at 6.6 and 33 µmol/L inhibited cell growth by 25 and 50%, respectively. Retinyl acetate also induced characteristics of secretory cells in the 100 WT squamous cells and enhanced these features in the T8 cells. This effect was evidenced by an increase in the synthesis and secretion of mucins and reduction of cell stratification (Marchok *et al.*, 1981).

Organ cultures of tracheas from hamsters fed a vitamin A-deficient diet underwent squamous metaplasia and keratinization. Retinyl acetate reversed these metaplastic changes (Clamon *et al.*, 1974; Newton *et al.*, 1980). This organ culture was used to screen numerous retinoids for reversal of keratinization. The concentration required for 50% effectiveness (ED_{50}) was 0.7 nmol/L for retinol and 1 nmol/L for retinyl acetate. In contrast, retinoic acid and some

synthetic retinoids had greater potency than retinol (e.g., $ED_{50} < 0.03$ nmol/L) (Newton *et al.*, 1980). Cigarette smoke condensate increased cell proliferation in hamster tracheal organ culture, whereas retinol suppressed this hyperplastic effect (Rutten *et al.*, 1988a).

Not only organ culture but also primary epithelial cells derived from hamster trachea have been studied for the effects of vitamin A and found to respond to vitamin A deficiency and supplementation in a similar fashion as in organ culture and *in vivo*. Thus, hamster tracheal epithelial cells maintained in primary culture in serum-free medium (vitamin A-deficient state) appeared squamous-like and stratified and produced a more complex keratin pattern (keratins 5–7, 8, 14, and 17–19) than cells cultured in vitamin A-supplemented medium, which formed a simple cuboidal epithelium and produced only four simple epithelial keratins (7, 8, 18 and 19) (Edmondson *et al.*, 1990). Further studies in this cell system have shown that hamster tracheal epithelial cells maintained in vitamin A-deficient medium had decreased expression of differentiation-related keratins (5, 6, 14 and 17) after exposure to the carcinogen BP. In contrast, cells maintained in medium containing 1 μmol/L retinol showed no effect of BP on the keratin expression pattern, indicating a protective effect of vitamin A (Edmondson & Mossman, 1991). Studies with primary hamster tracheal epithelial cells in serum-free, hormone-supplemented medium showed that cigarette smoke condensate inhibited dye-coupled intercellular communication between the epithelial cells, whereas retinol given to the cells at pharmacological concentrations concurrently with the condensate counteracted the inhibitory effect of cigarette smoke condensate on intercellular communication (Rutten *et al.*, 1988b). This observation suggests that maintenance of gap junctional communication by retinol may contribute to chemopreventive activity.

Wu and his colleagues investigated the effects of vitamin A on human and non-human primate tracheobronchial epithelium. They found that vitamin A inhibited the synthesis of keratins 5, 6, 14, 16 and 17 and stimulated keratins 7, 8, 10, 13, 15, 18 and 19 (Huang *et al.*, 1994) and also increased the production of hyaluronate (Wu & Wu, 1986). Optimal conditions for the expression of mucociliary function (ciliogenesis or mucin secretion) were achieved when early-passage human tracheobronchial cells were transplanted onto tracheal grafts, not onto plastic plates. Although cell attachment and proliferation were stimulated when tissue culture plates were coated with collagen gel, the expression of mucous cell function in culture occurred only when retinol was present in the medium (Wu *et al.*, 1990).

Different mechanisms mediate the effects of retinol on the levels of differentiation markers. Whereas the expression of a squamous cell marker, namely small proline-rich protein gene (*spr1*) (An *et al.*, 1993) and several keratins (Huang *et al.*, 1994) was down-regulated post-transcriptionally by retinol, the expression of mucin 2 (*MUC2*) gene was suppressed at the level of transcription (An *et al.*, 1994). The effects of retinol on the growth of human tracheobronchial epithelial cells in serum-free medium were variable, depending on the presence or absence of epidermal growth factor (EGF). In the absence of EGF, retinol caused a dose-dependent inhibition of growth, whereas in the presence of EGF, retinol was slightly growth-stimulatory (Miller *et al.*, 1993). Further studies suggested that the cells secreted a transforming growth factor (TGF)-α-like mitogen in the absence of retinol and that retinol suppressed the production of this factor (Miller *et al.*, 1993). Retinol also suppressed the growth of a cell line derived from human fetal lung in collagen gel culture (Emura *et al.*, 1988).

Only a few studies have analysed the effects of vitamin A on distal airway epithelial cells. These cells exhibit extensive proliferative capacity and have the potential to differentiate into mucociliary or epidermoid phenotype. Small amounts of serum induce undifferentiated cells to become ciliated and non-ciliated secretory cells, whereas they differentiate into epidermoid cells in retinol-free or serum-free medium (Kitamura *et al.*, 1990). Shibagaki and co-workers (1994) found that retinol enhanced the outgrowth of epithelial cells from explants of human peripheral lung tissue at 0.01 and 0.1 μmol/L, whereas 10 μmol/L retinol inhibited

the growth. However, long-term growth on a plastic surface was suppressed by retinol even at 0.1 µmol/L. The efficiency of colony formation by these epithelial cells on a fibroblast feeder layer was reduced by retinol at both 0.1 and 10 µmol/L. DNA synthesis was also inhibited by retinol. These findings highlight the role of retinol in regulating the proliferation and differentiation of tracheobronchial and distal lung epithelial cells, which may be the basis for the chemopreventive effects of retinyl palmitate *in vivo* in animal models (Saffiotti *et al.*, 1967) and human patients (Pastorino *et al.*, 1993).

Most lung cancer cell lines examined are resistant to growth inhibition by retinoids (Geradts *et al.*, 1993). However, a two-day pretreatment with retinyl acetate inhibited the migration and invasion of A549 human lung carcinoma cells *in vitro* through a human amnion basement membrane and the degradation of proline-labelled basement membrane components by 50% at non-cytotoxic concentrations of 0.09 and 3 µg/mL, respectively. This effect was accompanied by a significant decrease in type IV collagenase activity without a change in tissue transglutaminase activity. These findings suggest that retinol may suppress the expression of invasive phenotype in carcinoma *in situ* and thereby might prevent the development of a malignant lesion from a premalignant one (Fazely *et al.*, 1988).

Effects of vitamin A on normal and malignant oral cavity epithelial cells have not been investigated extensively, although animal studies have demonstrated chemopreventive effects of retinoids (Shklar *et al.*, 1980; Inoue *et al.*, 1993) and clinical trials have demonstrated that premalignant lesions and second primary tumours in the head and neck regions are prevented by 13-*cis*-retinoic acid (Hong *et al.*, 1995). Nonetheless, retinyl acetate caused a 50% reduction in the survival rate of a human tongue cancer cell line (Inoue *et al.*, 1995) and a maxillary cancer cell line (Yamamoto *et al.*, 1996) in a colony-forming assay in monolayer cell cultures at concentrations of 60 and 28 µg/mL, respectively. Cell cycle analysis demonstrated an increase in G_0/G_1 phase in the presence of vitamin A, which indicated that growth inhibition may be the result of suppression of DNA synthesis. Effects on squamous differentiation were noted in head and neck squamous carcinoma cell line 1483. Cells grown in delipidized serum depleted of endogenous retinoids expressed keratins with molecular weights of 67, 56, 54, 52, 48, 46 and 40 kDa (Poddar *et al.*, 1991). In contrast, cells grown in medium with 10% fetal bovine serum, which contained about 0.06 µmol/L retinol, expressed much less 67 kDa keratin, whereas the levels of keratins of molecular weight 46 and 48 kDa were lower and the amounts of the 52 and the 40 kDa keratins were higher than those expressed in cells grown in delipidized serum. Thus, vitamin A present in serum modulated the expression of several keratins in the 1483 cells (Poddar *et al.*, 1991).

(c) Normal and malignant leukocytes

Retinol or retinyl acetate modulate the proliferation and differentiation of normal and malignant haematopoietic cells. Retinyl acetate (3 µmol/L) stimulated clonal growth of committed myeloid stem cells from normal human bone marrow to form colonies of granulocytes and/or macrophages in soft agar in the presence but not in the absence of colony-stimulating activity factor (CSF). These retinoids potentiated the response of the stem cells to the growth factor CSF. Interestingly, at 30 µmol/L all these retinoids inhibited colony formation (Douer & Koeffler, 1982).

B-cells deprived of retinol in cell culture die within days by a process that is neither classical apoptosis nor the result of cell cycle arrest. The cells can be rescued by physiological concentrations of retinol and retinal, but not by retinoic acid. Retinol is not metabolized to retinoic acid in these cells. However, it is converted into several metabolites, one of which has the ability to sustain B-cell growth in the absence of an external source of retinol (Buck *et al.*, 1990, 1991b). The active metabolite of retinol in B-lymphocytes and other cell lines is 14-HRR and it mediates the effect of retinol on cell growth (Buck *et al.*, 1991a). Thus, a distinct retinoid signalling pathway may regulate the growth of some cells. In addition to being an essential cofactor for growth of B-lymphocytes in culture, retinol is also required for activation

of T-lymphocytes by antigen receptor-mediated signals. 14-HRR has been implicated as the intracellular mediator of this effect. Anhydroretinol, a retinol metabolite derivative with a retro-structure produced in activated human B-lymphocytes, reversibly inhibits retinol- and 14-HRR-dependent effects and blocks B-lymphocyte proliferation as well as activation of resting T-lymphocytes (Buck *et al.*, 1993). Anhydroretinol given to T-cells in the absence of 14-HRR induces rapid cell death which does not require messenger RNA and protein synthesis. The data suggest that retro-retinoids act in the cytoplasm as second messengers like diacyl glycerol or ceramide and do not require modulation of gene expression by nuclear retinoid receptors (O'Connell *et al.*, 1996).

Breitman and his collaborators have demonstrated that retinoic acid (1 µmol/L, 6 days) can induce the differentiation of >90% of HL-60 myeloid leukaemia cells into granulocytes. Retinol and retinyl acetate at the same concentration caused <20% of HL-60 cells to differentiate. In contrast, 0.1 µmol/L of retinoic acid induced the differentiation of 50% of the cells (Breitman *et al.*, 1980). In HL-60 myeloid leukaemia cells, retinol is metabolized to 14-HRR, anhydroretinol, retinoic acid and retinyl esters. Exogenous application of the retinol metabolites in retinol-depleted serum-free cultures of HL-60 allowed the identification of unique cellular functions for each metabolite: 14-HRR is a growth factor for HL-60; anhydroretinol is a functional antagonist of 14-HRR with growth-inhibiting activity, and retinoic acid is a potent inducer of granulocyte differentiation accompanied by growth arrest (Eppinger *et al.*, 1993).

Retinol–RBP and chylomicron remnant retinyl esters in concentrations normally found in human plasma inhibit growth of normal human B-lymphocytes. Physiological concentrations of retinoic acid (about 30 nmol/L) were less active than physiological concentrations of retinol (about 3 µmol/L). Pharmacological concentrations of retinol and retinoic acid were more active than the concentrations normally found in plasma. Retinol (3 µmol/L) inhibited anti-IgM-mediated DNA synthesis by 78%. Furthermore, cells were blocked in the mid-G_1 phase of the cell cycle. The late activation markers (transferrin receptor expression and actinomycin D staining at 48 hours of stimulation) were markedly inhibited. After 48 h, retinol also reduced the interleukin-6 production that was induced either by anti-IgM or by interleukin-4. Retinoids reduced the formation of plaque-forming cells (i.e., Ig synthesis) (Blomhoff *et al.*, 1992).

(d) Untransformed and transformed fibroblasts

The anchorage-independent growth of human fibroblasts induced by platelet-derived growth factor (PDGF) or by basic fibroblast growth factor (bFGF) was inhibited by physiological concentrations of either retinol (0.5 µmol/L) or retinoic acid (1.0 nmol/L), but not by anhydroretinol (0.5 µmol/L). Retinol also reduced the frequency of anchorage-independent growth of the human fibrosarcoma-derived cell line, HT1080, which formed colonies in semi-solid medium without added growth factors. These results suggest that physiologically active retinoids suppress properties associated with transformation (Palmer *et al.*, 1989).

Treatment of postconfluent cultures of C3H 10T1/2 mouse fibroblasts with certain carcinogens has been shown to cause transformation to focus-forming cells. The development of this in-vitro cell transformation system has enabled investigators to examine the ability of vitamin A to intervene in such transformation. Retinyl acetate was found to be highly active in inhibition of MCA-induced neoplastic transformation of C3H 10T1/2 cells. When MCA-treated cultures were treated continuously with retinyl acetate (0.1 µg/mL), or for 24 hours with 2.5 µg/mL starting seven days after MCA exposure, or after delaying retinyl acetate treatment up to three weeks after MCA exposure, neoplastic transformation was inhibited by 100%, 70% and 80% respectively (Merriman & Bertram, 1979). The efficacy of retinol and retinal in this system was similar to that of retinyl acetate. The ability of retinyl acetate to inhibit transformation even when added one week after the carcinogen excluded an effect on carcinogen metabolism, on the initiation phase of carcinogenesis or on the fixation of the carcinogenic damage. The effect of retinyl acetate on trans-

formation was reversible, as transformed foci began to appear after the retinoid was removed for three to five weeks, suggesting that, when present, retinyl acetate suppressed the progression of preneoplastic cells to fully neoplastic cells. Further, fully transformed cell lines derived from cultures exposed to MCA appeared to be resistant to growth inhibition by retinyl acetate (0.1 µg/mL) when cultured on confluent monolayers of 10T1/2 cells, suggesting that the effect of retinyl acetate in this system was limited to preneoplastic cells (Merriman & Bertram, 1979; Mordan *et al.*, 1982). Subsequent studies have demonstrated that retinyl acetate increased the degree of adhesion of C3H 10T1/2 cells to a plastic substrate (Bertram, 1980) and that their ability to communicate via gap junctions, which was reduced by exposure to MCA, was enhanced by retinyl acetate at the same concentrations that inhibited neoplastic transformation (Hossain *et al.*, 1989). More recently, it was found that retinoids induce the gap junctional protein connexin 43 (Rogers *et al.*, 1990). These observations suggest that the chemopreventive effects of retinoids may be explained partially by enhanced gap junctional communication.

Cultured C3H 10T1/2 cells transfected with the plasmid pdPBV-1 harbouring bovine papillomavirus (BPV) DNA were used for assessing *in vitro* the tumour-promoting and chemopreventive activities of various agents. The exposure of such cells to extracts of areca nut (used in betel quid), which have been linked to the high incidence of precancerous oral lesions and oral cancers in India, enhanced the formation of BPV DNA-induced transformed foci approximately tenfold. The addition of retinol to the areca nut extract inhibited its tumour-promoting effect in a dose-dependent manner, completely abolishing the promoting activity at a dose of 1 µmol/L. This effect was proposed to be one of the mechanisms by which vitamin A intake could reduce oral cancer incidence among chewers of areca nut/tobacco mixtures and of the chemopreventive effect of vitamin A administered to betel quid chewers (Stich & Tsang, 1989).

The transformation of C3H 10T1/2 mouse fibroblasts was shown to be induced in density-arrested initiated cells by PDGFs in serum and was correlated with the mitogenic response of the preneoplastic cells to PDGF or EGF. The stimulation of DNA synthesis and cell division in normal and carcinogen-treated C3H 10T1/2 fibroblasts by serum after density-dependent growth arrest was inhibited by retinyl acetate to the same extent that neoplastic transformation was inhibited by this retinoid. On the basis of these data, Mordan (1989) suggested that the inhibition of neoplastic transformation by retinol is the result of blocking the G_0 to G_1 transition in the mitotic response of initiated cells to platelet growth factors which act as autocrine or endogenous promoters of transformation.

(e) Mammary cancer cell lines

Several studies have demonstrated that vitamin A can modulate the proliferation and differentiation of rat and human mammary cancer cells. In a rat mammary cancer stem-like cell line (Rama 25), differentiation to alveolar-like cells is evidenced by the increase in production of domes (hemispheric blisters) in the cell monolayer and the appearance of immunoreactive casein in the tissue culture medium. This differentiation was enhanced by retinol and retinyl acetate (0.04 to 4 µmol/L) in the presence of the hormones prolactin, hydrocortisone, insulin and 17β-estradiol. These retinoids also caused a reduction in the rate of DNA synthesis. Pretreatment of the Rama 25 cells with retinyl acetate before injection into immunocompromised young female nu/nu (nude) mice decreased the incidence of tumours compared with injections of untreated cells. The findings suggest that the ability of retinoids to suppress rat mammary gland carcinogenesis may be due to their differentiation-inducing properties and their ability to suppress DNA synthesis (Rudland *et al.*, 1983). Indeed, Mehta and Moon (1980) observed suppression of DNA synthesis in mammary cells from rats exposed to DMBA or MNU and placed on a diet supplemented with retinyl acetate, as compared to cells from rats exposed to the carcinogens but fed a diet without retinyl acetate supplement. There was no effect of retinyl acetate on DNA synthesis in cells from rats that were not

exposed to carcinogens, suggesting a selective effect on initiated or transformed cells.

Inhibition of the growth of human breast cancer cell lines by retinol has been reported by several groups. Ueda *et al.* (1980) observed a good correlation between the ability of retinol to inhibit cell proliferation and the synthesis of DNA (thymidine incorporation) in MCF-7 mammary carcinoma cells in culture, suggesting that suppression of DNA synthesis is the primary cause of cell growth inhibition. Other studies have shown that retinol inhibited the anchorage-dependent and anchorage-independent growth of the human mammary carcinoma cell line MDA-MB-231. Clones resistant to growth inhibition by retinol were isolated from this cell line in soft agar. Because the clones were still susceptible to growth inhibition by all-*trans*- and 13-*cis*-retinoic acid, it is possible that metabolism of retinol to all-*trans*-retinoic acid was absent in these cells (Halter *et al.*, 1990).

Although in many cell systems, retinol is metabolized to retinoic acid, which is presumed to be the ultimate mediator of the effects of retinol on gene expression and the changes in cell growth and differentiation that these lead to, there are cell types that do not metabolize retinol to retinoic acid. For example, retinoic acid inhibits the growth of 'normal' human breast epithelial cell strains AD074 and MCF10A and the estrogen receptor-positive (ER+) breast cancer cell lines MCF-7 and T47D. However, these cells do not metabolize retinol to retinoic acid, but instead form 4-oxoretinol. Interestingly, exogenous 4-oxoretinol inhibits the growth of these cells. Further, 4-oxoretinol also inhibits the growth of the ER– breast cancer lines MDA-MB-231, MDA-MB-468 and BT20, although these cells do not metabolize retinol to 4-oxoretinol (Chen *et al.*, 1997b). Because 4-oxoretinol has been shown to activate nuclear retinoic acid receptors to mediate gene transcription, it is possible that the growth inhibitory effects of both retinol and 4-oxoretinol are mediated by alterations in gene expression (Achkar *et al.*, 1996).

Some additional clues on the mechanism of breast cancer growth suppression by retinol have come from studies that showed that pretreatment of two human mammary carcinoma cell lines (retinoid-sensitive T47D and retinoid-resistant MDA-MB-468) for 48 hours with retinol resulted in inhibition of TGFα stimulation of growth. In the T47D cell line, the mechanism appeared to be loss of TGFα-induced stimulation of the EGF receptor substrate, phospholipase C-g 1. Alteration of phospholipase C-g 1 activity was not responsible for the inhibition of cell growth seen in the presence of retinol in the absence of TGFα stimulation. In the MDA-MB-468 cell line, pretreatment with retinol resulted in a decrease in tyrosine phosphorylation of the EGF receptor (Halter *et al.*, 1993). Thus, retinol may interfere with EGF receptor-mediated mitogenic signalling.

(f) Prostate cells

Mouse prostate gland exposed to MCA or maintained in organ culture in vitamin A-deficient medium undergoes hyperplasia and squamous metaplasia. These changes are reversed after addition of retinol to the medium and prevented in organ cultures exposed to both carcinogen and vitamin A (Lasnitzki, 1955, 1962; Lasnitzki & Goodman, 1974).

Retinoic acid was more effective in this organ culture system than retinol. In tissues, most of the endogenous retinoic acid is formed from retinol and could mediate the effects of vitamin A. Support for this conclusion comes from studies that demonstrated the ability of liarozole, an inhibitor of cytochrome P450 enzymes, to suppress keratinization of a rat prostate carcinoma. Further, *in vivo*, therapy with liarozole increases plasma and tissue levels of retinoic acid, which may be a contributing factor in its retinoid-mimetic effects. In the rat Dunning prostate cancer models, liarozole inhibited the growth of androgen-independent as well as androgen-dependent carcinomas relapsing after castration. Concurrently, changes in the pattern of cytokeratins characteristic of increased differentiation were observed (De Coster *et al.*, 1996). More importantly, liarozole inhibited *in vitro* the secretion of type IV collagenase and invasion through Matrigel in a Boyden chamber by the androgen-independent PC-3ML-B2 human prostatic

carcinoma clone without inhibiting cell proliferation and cell attachment. *In vivo*, liarozole treatment increased the retinoic acid levels in tumours that developed in severe-combined immunodeficient (SCID) mice, blocked type IV collagenase production in established subcutaneous tumours and reduced the growth of bone metastases of the PC-3ML-B2 cells (Stearns *et al.*, 1993). All of these effects are presumed to be caused by the accumulation of retinoic acid derived from endogenous intracellular stores of vitamin A.

(g) Bladder cancer cells

Vitamin A deficiency in the rat has been reported to lead to rapid growth and keratinization *in vivo* and in organ culture, suggesting a role for vitamin A in the control of differentiation of bladder epithelial cells. Indeed, retinol increased the level of alkaline phosphatase, which is decreased in serum-depleted medium, but did not suppress hyperplasia (Reese & Politano, 1981). The rat Nara Bladder Tumor No. 2 (NBT II) cell line, established from a urinary bladder carcinoma in the Wistar rat, underwent squamous differentiation including the formation of keratin pearls when cultured as multicellular aggregates. Supplements of vitamin A in the form of retinyl palmitate (1 to 10 IU/mL) to the medium prevented keratinization but did not inhibit aggregate formation. Vitamin A also enhanced the number of cells engaged in DNA synthesis. The inhibition of keratinization was reversible; after vitamin A was removed from the medium, the cells in the aggregates resumed the process of keratinization (Toyoshima & Leighton, 1975; Tchao, 1980). The ability of retinol to prevent keratinization in six transformed rat bladder cell lines was related to the expression of cellular RBP (Kawamura & Hashimoto, 1980).

(h) Colon cancer cells

Retinyl palmitate at concentrations of 0.1 µmol/L and above decreased by about 80% DNA synthesis measured by thymidine incorporation after one day of treatment of a rat Prob colonic tumour cell line. The treatment also induced intestinal-type alkaline phosphatase expression, and increased slightly the proportion of floating apoptotic cells as indicated by DNA ladder formation and chromatin condensation (Maziere *et al.*, 1997).

Retinol added to freshly passaged cultures of FHC, a human colonic epithelial cell line, inhibited proliferation in dose-dependent fashion. Polyamine content, specifically the spermidine content and the spermidine/spermine ratio, decreased in response to culture with retinol, suggesting that ornithine decarboxylase (ODC) was inhibited (Higuchi & Wang, 1995). The same investigators also found that physiological concentrations of retinol stimulated the induction of NAD(P)H:quinone reductase, a phase II detoxifying enzyme, in human Colo205 colon carcinoma cell line (Wang & Higuchi, 1995). These findings suggest that anticarcinogenic effects of retinol in colon epithelium may be mediated by induction of detoxifying enzymes, decreased cell proliferation, enhanced differentiation and apoptosis.

(i) Transformed ovarian cells

Studies with Chinese hamster ovary cells have demonstrated that retinol blocks cell cycle progression in the G_1 phase of the cell cycle, and that there is a concentration-dependent inhibition of ODC induction. Retinol inhibited the induction of ODC activity only when added in the first 2–3 h of G_1 progression. Because polyamines required for normal cellular growth are produced by ODC, it was postulated that the antiproliferative properties of retinoids are related to their ability to inhibit ODC expression by inhibition of ODC mRNA synthesis (Russell & Haddox, 1981). This finding may be relevant also to suppression of carcinogenesis, because ODC is increased by tumour-promoting agents and suppressed by retinoids in mouse skin (Verma *et al.*, 1979) and in rat liver (Van Rooijen *et al.*, 1984).

(j) Embryonal teratocarcinoma cells

Retinol induced the differentiation of several mouse embryonal teratocarcinoma cell lines (certain F9, OC15S1 and Nulli-SCC1) in culture, albeit with a lower potency than retinoic acid (Eglitis & Sherman, 1983). However, its physiological concentration is also much higher than that of retinoic acid and therefore retinol may

have an important function in modulating differentiation *in vivo*. Recently, it was found that F9 cells metabolize retinol to 4-oxoretinol and that the latter can activate nuclear retinoic acid receptors to enhance transcription of specific genes and thereby cause cell differentiation. This constitutes a novel pathway for signalling by retinol without metabolism to all-*trans*- or 9-*cis*-retinoic acid (Achkar *et al.*, 1996).

(k) Miscellaneous normal and tumour cells

Previous studies have demonstrated that retinoic acid can induce the expression of TGFβ *in vivo* in vitamin A-deficient rat tissues (Glick *et al.*, 1991). This finding suggested indirectly that retinol may be involved in normal regulation of TGFβ expression in certain tissues. However, it was reported recently that retinol can activate a latent form of TGFβ in isolated avian osteoclasts (Bonewald *et al.*, 1997). The effect of retinol was restricted to mature osteoclasts and was not observed in osteoclast precursors. The mechanism of activation is not known, but it appears to be independent of the plasmin–plasminogen activator pathway. Because TGFβ is a multifunctional cytokine, which is found in many tissues in a latent form, its activation by retinol may be an important effect if reproduced in other cell types, especially in view of the growth inhibitory effect of TGFβ on many epithelial cancer cells.

Retinol inhibited the growth of three cell lines derived from a single Shope carcinoma and differing in their degree of differentiation. The growth-retarding effect was reversible in the undifferentiated subline upon removal of retinol from medium, but the two differentiated sublines did not revert. A seven-day pretreatment of the differentiated sublines with retinol decreased the tumorigenic potential of the differentiated sublines, whereas the potential of the undifferentiated subline was only slightly reduced. These results suggest that retinol may be effective against differentiated squamous cell carcinomas, but not against undifferentiated tumours (Isono & Seto, 1991).

Vitamin A added to the culture medium at a 50 μmol/L concentration caused a 60% inhibition of DNA synthesis in melanoma (SK Mel 28) and cervical carcinoma (HeLa) cell lines; the inhibition was reversible and this treatment did not select for retinol-resistant clones (Ferrari *et al.*, 1983). The inhibitory effect of retinol on HeLa cells was correlated with inhibition of plasma membrane NADH oxidase activity, an activity which has been implicated in the progress of cell proliferation (Dai *et al.*, 1997).

(l) Molecular mechanisms of gene regulation by retinol

There is ample evidence that the major mechanism by which retinoids, including retinol, exert their various effects on cell growth and differentiation is via modulation of gene expression (DeLuca, 1991; Gudas *et al.*, 1994). Nuclear retinoic acid receptors, which are members of the steroid hormone receptor superfamily, function as ligand-activated trans-acting transcription modulating factors and have been implicated as the proximal mediators of the effects of retinoids on gene expression (Chambon, 1996; Mangelsdorf & Evans, 1995; Mangelsdorf *et al.*, 1994).

Retinol can regulate the transcription of genes by serving as a precursor for metabolites that activate nuclear retinoid receptors. There are at least two ways by which retinol can do this. One pathway requires the formation of all *trans*- and 9-*cis*-retinoic acid. Both isomers can bind to nuclear retinoic acid receptors (RAR) α, β and γ, whereas 9-*cis*-retinoic acid can also bind to retinoid X receptors (RXR) α, β and γ (Chambon, 1996; Mangelsdorf & Evans, 1995; Mangelsdorf *et al.*, 1994).

Evidence to support the above pathway is described below. Studies with cultured human keratinocytes have demonstrated that the biological activity of retinol in activation of gene expression (determined as reduction of type I transglutaminase and stimulation of a β-retinoic acid response element $(RARE)_3$-driven reporter gene activity) requires receptors, because dominant-negative mutant receptor cotransfection eliminated the effects of retinol. Further, inhibition by citral of metabolism of all-*trans*-retinol to all-*trans*-retinoic acid reduced β $RARE_3$-tk-CAT activity by 98%. These data indicate that retinol-induced responses in human keratinocytes are mediated by its metabolite retinoic acid, which functions as a

ligand to activate nuclear retinoic acid receptors (Kurlandsky *et al.*, 1994). Cope and Wille (1989) reported that treatment of human malignant keratinocytes with 30 µmol/L antisense oligodeoxynucleotides corresponding to human nuclear RAR-α blocked the induction of alkaline phosphatase by retinol in these cells. It is noteworthy that there is no simple relationship between the expression of nuclear retinoid receptors and response of cells to growth-inhibitory effects of retinol.

Another pathway is based on the reported ability of the retinol metabolite 4-oxoretinol at low doses (0.1 and 1 nmol/L) to activate the transcription of a reporter gene driven by RARE via RARs (Achkar *et al.*, 1996).

A nuclear receptor-independent mechanism has also been suggested to account for the activity of the retinol metabolite anhydroretinol, which acts without requiring synthesis of either mRNA or protein and appears to be able to affect cell growth and differentiation by a still unknown process (O'Connell *et al.*, 1996).

It is of interest to note that vitamin A deficiency (Haq *et al.*, 1991; Verma *et al.*, 1992), treatment with tumour promoters (Kumar *et al.*, 1994) and the development of premalignant and malignant lesions *in vivo* (Darwiche *et al.*, 1995; Lotan *et al.*, 1995; Xu *et al.*, 1994, 1997) are all associated with decreased expression of certain nuclear retinoid receptors *in vivo*. The expression of these receptors can be restored to normal levels by supplementation with retinoic acid (Haq *et al.*, 1991; Lotan *et al.*, 1995).

4.2.3.2 In-vitro inhibition of genetic and related effects

Many studies have dealt with the antimutagenic effects of retinol, retinal, retinoic acid and retinyl esters in short-term tests using prokaryotes or eukaryotes under in-vitro conditions. Reviews (Odin, 1997) and antimutagenicity profiles have been published in recent years (Brockman *et al.*, 1992; Waters *et al.*, 1990, 1996a, b). Such profiles are presented in Figures 6–8.

The results of studies using *Salmonella typhimurium* strains as targets of genotoxicity are summarized in Table 26. One study (Okai *et al.*, 1996) evaluated inhibition of mutagen-induced *umu* C gene expression in strain TA1535/- pSK 1002. Retinol, retinyl acetate, retinyl palmitate and retinoic acid inhibited the induction of *umu* C gene expression by the heterocyclic amine 3-amino-3,4-dimethyl-5*H*-pyrido[4,3-*b*]indole (Trp-P-1). In contrast, retinol, retinyl acetate and retinoic acid were ineffective when *umu* C gene expression was induced by the direct-acting mutagens adriamycin and mitomycin C.

A number of studies were performed with the Ames *Salmonella*/microsome test. Neither retinol nor retinoic acid affected the mutagenicity of hydrogen peroxide in strain TA104 (Han, 1992). Retinyl acetate decreased the mutagenicity of cysteine, occurring at high, non-physiological concentrations in strain TA102 (Stark *et al.*, 1994).

Vitamin A and its derivatives failed to inhibit the mutagenicity of certain direct-acting mutagens, as shown, for example, by the inactivity of retinyl palmitate and retinoic acid towards 4-nitroquinoline 1-oxide mutagenicity in TA100 (Camoirano *et al.*, 1994), and of retinol towards that of either adriamycin in TA98 (Baird & Birnbaum, 1979), mitomycin C in TA102, irrespective of the presence of rat liver S9 fractions (Qin & Huang, 1985), diepoxybutane in TA1535 (Busk & Ahlborg, 1980), photoactivated 2-azido-9-fluorenone oxime in TA1538 (White & Rock, 1981) and 4-nitro-*o*-phenylenediamine in TA98 (Balbinder *et al.*, 1983). The mutagenic potency of the last compound was decreased in the presence of S9 mix, and the effect was not influenced by retinol (Balbinder *et al.*, 1983). In the case of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG), retinol and retinal displayed antimutagenic activity in strain TA100, which contrasted with the lack of activity of retinoic acid, retinyl acetate and retinyl palmitate (Shetty *et al.*, 1988). On the other hand, the direct mutagenicity of nitro compounds (2-nitrofluorene, 1-nitropyrene and 3-nitrofluoranthene) in TA98 was decreased by retinol and retinoic acid and, with greater potency, by retinal and retinyl palmitate (Tang & Edenharder, 1997). Moreover, retinol significantly inhibited the direct mutagenicity in TA100 of methylazoxymethanol, a metabolite of 1,2-dimethylhydrazine, but the effect was not dose-

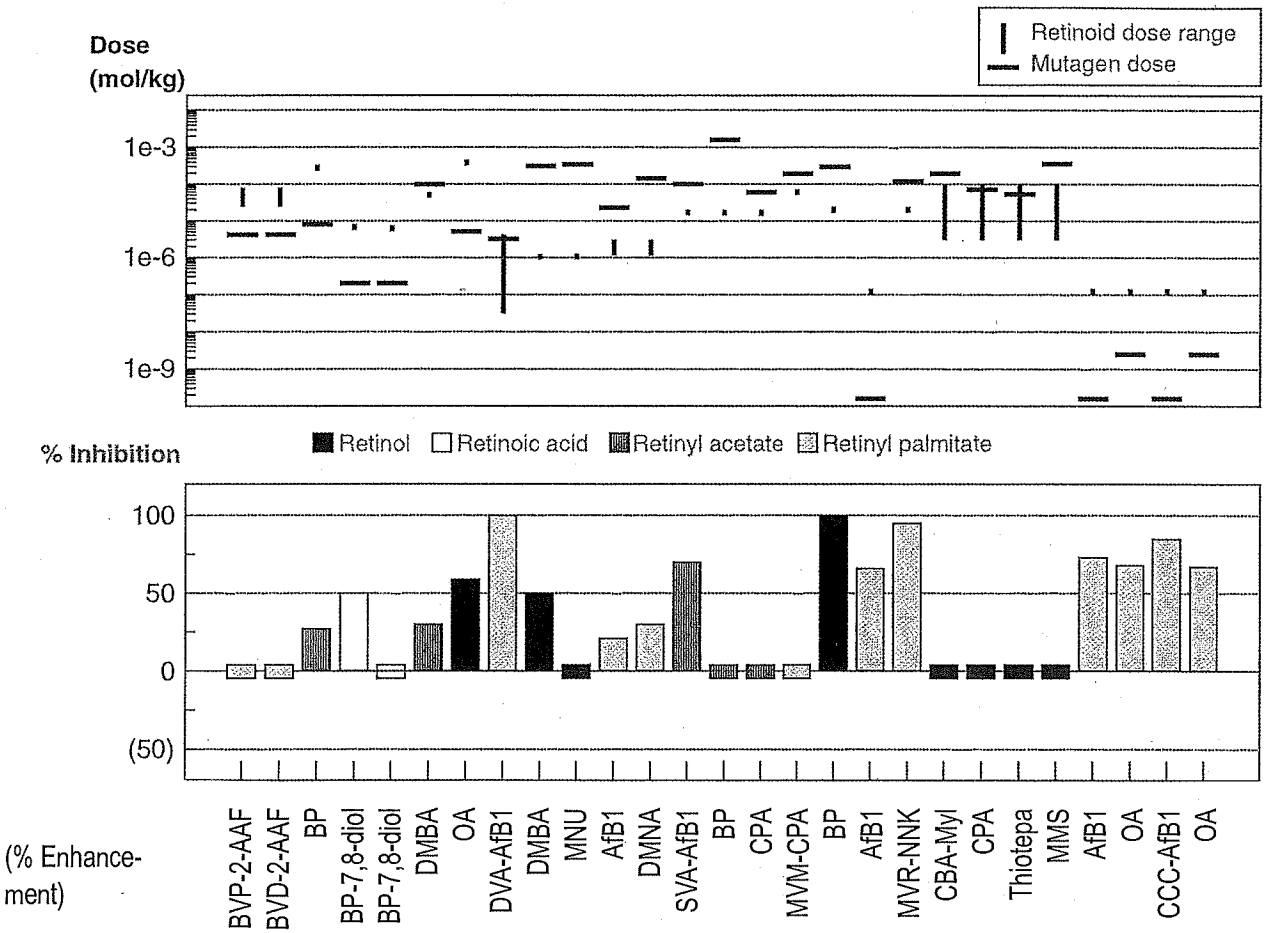

Figure 6. The antimutagenicity profile shows the performance of retinol, retinoic acid and retinyl esters to modulate genotoxin-induced effects in mammals *in vivo*.

The profile is organized using the test codes and chemical abbreviations to identify each item across the profile, and only the first occurrence of each test code is given. In the upper panel, doses from the data in Table 25 are plotted in molar units for the genotoxins and the retinoids. In the lower panel positive values are the maximum percent inhibition of the genotoxin-induced effects, negative values are the maximum percent enhancement of the effects, and values equal to zero indicate that no significant difference was observed relative to the induced effects. A brief description of the antimutagenicity profile methodology and test code definitions are given in the appendices. Abbreviations: 2-acetylaminofluorene, 2-AAF; aflatoxin B_1, AfB1; benzo[*a*]pyrene, BP; (7S,8S)-dihydroxy-7,8-dihydrobenzo[*a*]pyrene, BP-7,8-diol; cyclophosphamide, CPA; 7,12-dimethylbenz[*a*]anthracene, 7,12-DMBA; dimethylnitrosamine, DMNA; methyl methanesulfonate, MMS; *N*-methylnitrosourea, MNU; busulfan (myleran), Myl; 4-(*N*-nitrosomethylamino)-1-(3-pyridyl)-1-butanone, NNK; ochratoxin A, OA.

dependent (Tavan *et al.*, 1997). Retinol weakly inhibited the direct mutagenicity in TA98 of complex mixtures, including coal dust, diesel emission particles, tobacco snuff and airborne particles, as well as the S9-mediated mutagenicity of fried beef extracts (Ong *et al.*, 1989).

Retinol, retinal, retinoic acid, retinyl acetate and retinyl palmitate were consistently effective in inhibiting the S9-mediated mutagenicity of aflatoxin B_1 in TA98 and TA100 (Busk & Ahlborg, 1980; Raina & Gurtoo, 1985; Qin & Huang, 1985; Bhattacharya *et al.*, 1987). Only in one study (Raina & Gurtoo, 1985) did retinoic acid fail to inhibit aflatoxin B_1 mutagenicity in TA100, although it was antimutagenic in TA98. Retinol, retinoic acid and retinyl acetate were approximately equipotent in a comparative study (Qin & Huang, 1985), whereas in another study (Bhattacharya *et al.*, 1987) the potency to decrease aflatoxin B_1

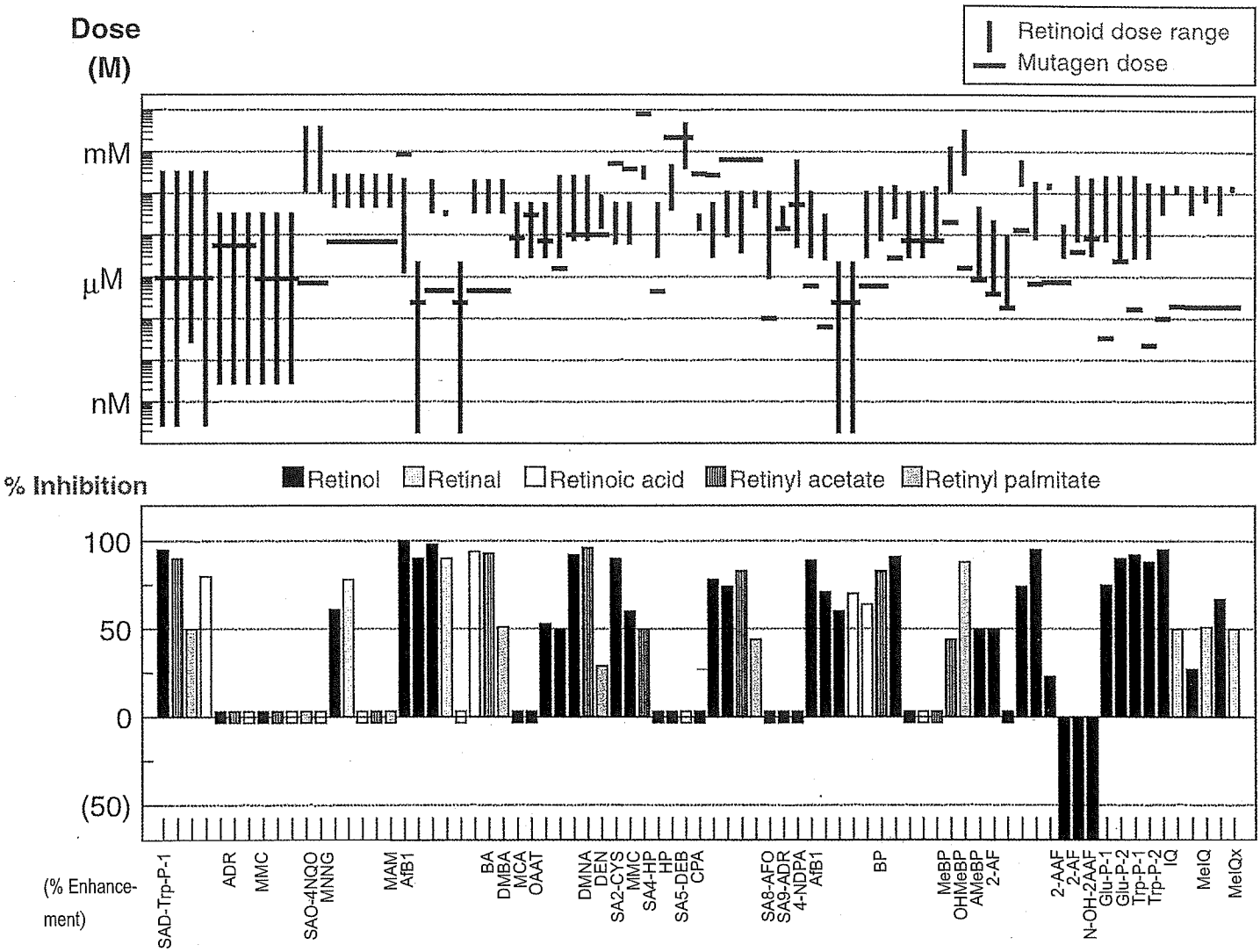

Figure 7. The antimutagenicity profile displays the performance of retinol, retinal, retinoic acid, and retinyl esters to modulate mutagen-induced effects in *Salmonella typhimurium* strains.
The profile is organized using the test codes and chemical abbreviations to identify each item across the profile, and only the first occurrence of each test code and/or mutagen is given. In the upper panel, doses from the data in Table 26 are plotted in molar units for both the mutagens and the retinoids. In the lower panel positive values are the maximum percent inhibition of the mutagen-induced effects, negative values are the maximum percent enhancement of the effects, and values equal to zero indicate that no significant difference was observed relative to the mutagen-induced effects. A brief description of the antimutagenicity profile methodology and test code definitions are given in the appendices. Abbreviations: 2-acetylaminofluorene, 2-AAF; 2-aminofluorene, 2-AF; adriamycin, ADR; aflatoxin B_1, AfB1; 2-azido-9-fluorenone; AFO; 6-acetoxymethylbenzo[*a*]pyrene, AMeBP; benz[*a*]anthracene; BA; benzo-[*a*]pyrene, BP, cyclophosphamide, CPA; cysteine, CYS; diepoxybutane, DEB; *N*-nitrosodiethylamine, DEN; 7,12-dimethyl-benz[a]-anthracene, DMBA, *N*-nitrosodimethylamine; DMNA; 2-amino-6-methylpyrido[1,2-*a*:3'2'-*d*]imidazole, Glu-P-1; 2-amino-dipyrido[1,2,-*a*:3',2'-*d*]imidazole, Glu-P-2; hydrogen peroxide, HP; 2-amino-3-methylimidazo[4,5-*f*]quinoline, IQ; methylazoxy-methanol, MAM; 3-methylcholanthrene, MCA; 6-methylbenzo[*a*]pyrene, MeBP; 2-amino-3,4-dimethylimidazo[4,5-*f*]quinoline, MeIQ; 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline, MeIQx; mitomycin C, MMC; *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine, MNNG; *N*-hydroxy-2-acetoaminofluorene, N-OH-2AAF; 4-nitro-*o*-phenylenediamine; 4NPDA; 4-nitroquinoline 1-oxide, 4-NQO; *o*-aminoazotoluene, OAAT; 6-hydroxybenzo[*a*]pyrene, OHMeBP; 3-amino-1,4-dimethyl-5*H*-pyrido[4,3-*b*]indole, Trp-P-1; 3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole, Trp-P-2.

mutagenicity ranked as follows: retinal > retinol and retinoic acid > retinyl acetate > retinyl palmitate. The antimutagenic effect was more evident when retinoic acid was added at the start of the metabolic activation reaction than when it was added after the reaction was terminated by menadione, suggesting an effect of retinoic acid on aflatoxin B_1 metabolism (Raina & Gurtoo, 1985). Inhibition of the metabolic activation of aflatoxin B_1 to mutagenic

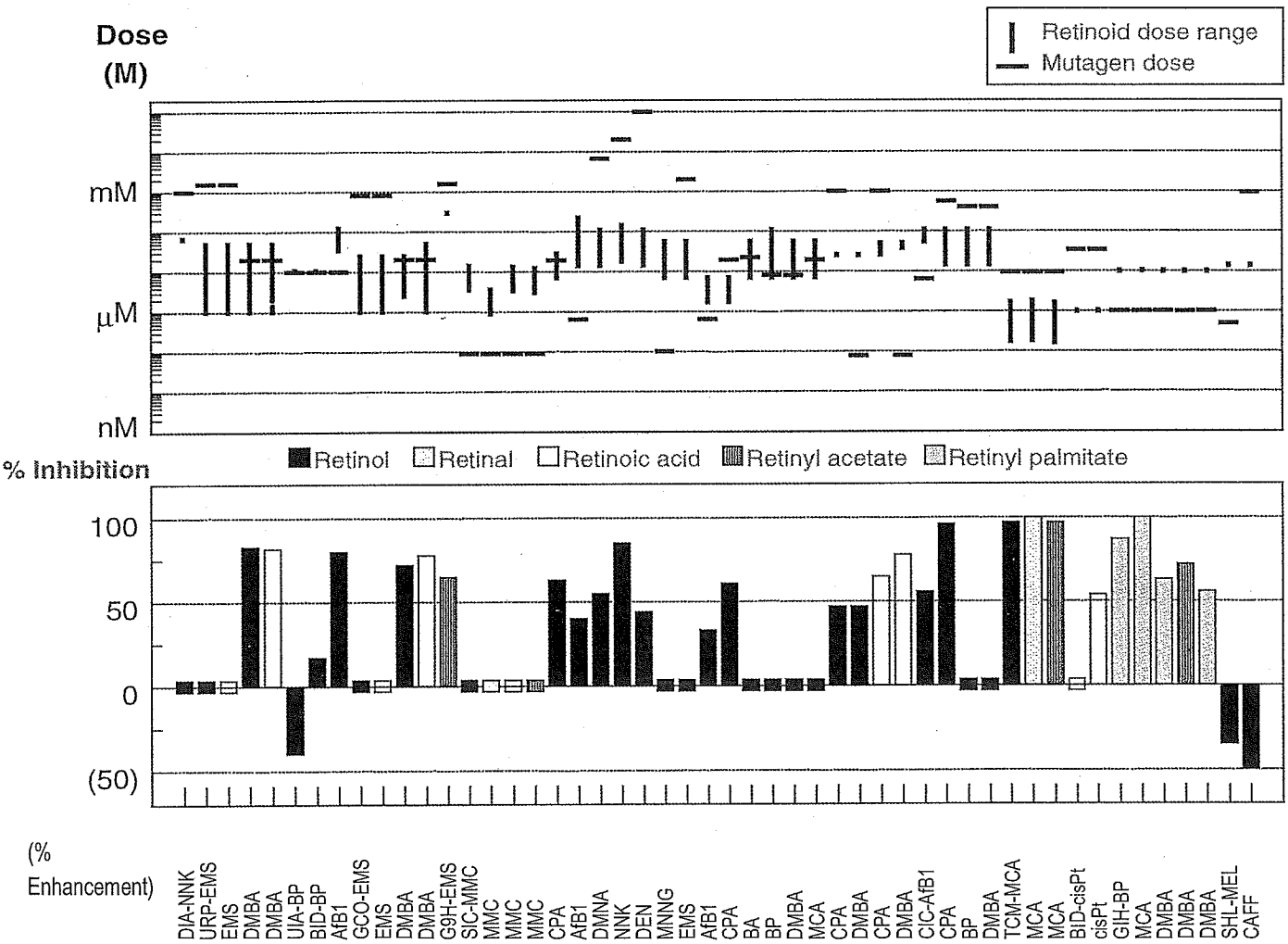

Figure 8. The antimutagenicity profile shows the performance of retinol, retinal, retinoic acid and retinyl esters to modulate genotoxin-induced effects in mammalian cells *in vitro*.

The profile is organized using test codes and chemical abbreviations to identify each item across the profile with only the initial occurrence of each test code given. In the upper panel doses from the data in Table 27 are plotted in molar units for both the genotoxins and retinoids. In the lower panel positive values are the maximum percent inhibition of the genotoxin-induced effects, negative values are the maximum percent enhancement of the effects, and values equal to zero indicate that no significant differences were observed relative to the induced effects. A brief description of the antimutagenicity profile methodology and test code definitions are given in the appendices. Abbreviations: aflatoxin B_1, AfB1; benz[*a*]anthracene, BA; benzo[*a*]pyrene, BP; caffeine, CAFF; cisplatin, cisPt; cyclophosphamide, CPA; *N*-nitrosodiethylamine, DEN; 7,12-dimethylbenz[*a*]anthracene, DMBA; *N*-nitrosodimethylamine, DMNA; ethyl methanesulfonate, EMS; 3-methylcholanthrene, MCA; melphalan, MEL; mitomycin C, MMC; *N*-methyl-*N*-nitro-*N*-nitrosoguanidine, MNNG; 4-(*N*-nitrosomethlyamino)-1-(3-pyridyl)-1-butanone, NNK.

metabolites was also observed when liver S9 fractions from mice maintained on a diet supplemented with either retinyl acetate (Qin & Huang, 1986) or retinyl palmitate (Decoudu *et al.*, 1992) were used, as compared to liver S9 fractions from mice maintained on a vitamin A-deficient diet.

Vitamin A and its derivatives were less consistently effective in decreasing the S9-mediated mutagenicity of polycyclic aromatic hydrocarbons. Thus, in a single comparative study in strain TA98 (Qin & Huang, 1985), retinol, retinoic acid and retinyl acetate failed to decrease the mutagenicity of BP, and retinol was also ineffective towards benz[*a*]anthracene and DMBA. A protective effect of retinol in this study was observed only towards MCA. There was no difference in the activation of BP to mutagenic metabolites in TA98 when liver S9 fractions from mice maintained on a vitamin

Table 26. Assay of retinol, retinal, retinoic acid and retinyl esters for the ability to inhibit standard mutagens in in-vitro systems using *Salmonella typhimurium* strains

End-point [a]	Code [a]	Modulator (tested doses) [b]	Mutagen (tested doses) [b]	*S. typhimurium* strain	S9 mix	Result [c]	Inhibitory dose (IDx) [d]	Reference
D	SAD	Retinol (0.0003–300 µM)	Trp-P-1 (0.2 µg/mL)	TA1535/ pSK1002	+	+	0.12 µM (ID50)	Okai *et al.* (1996)
D	SAD	Retinyl acetate (0.0003–300 µM)	Trp-P-1 (0.2 µg/mL)	TA1535/ pSK1002	+	+	0.3 µM (ID50)	Okai *et al.* (1996)
D	SAD	Retinyl palmitate (0.0003–300 µM)	Trp-P-1) (0.2 µg/mL)	TA1535/ pSK1002	+	+	60 µM (ID50)	Okai *et al.* (1996)
D	SAD	Retinoic acid (0.0003–300 µM)	Trp-P-1) (0.2 µg/mL)	TA1535/ pSK1002	+	+	0.54 µM (ID50)	Okai *et al.* (1996)
D	SAD	Retinol (0.003–30 µM)	Adriamycin (3 µg/mL)	TA1535/ pSK1002	–	–	NA	Okai *et al.* (1996)
D	SAD	Retinyl acetate (0.003–30 µM)	Adriamycin (3 µg/mL)	TA1535/ pSK1002	–	–	NA	Okai *et al.* (1996)
D	SAD	Retinoic acid (0.003–30 µM)	Adriamycin (3 µg/mL)	TA1535/ pSK1002	–	–	NA	Okai *et al.* (1996)
D	SAD	Retinol (0.003–30 µM)	Mitomycin C (0.3 µg/mL)	TA1535/ pSK1002	–	–	NA	Okai *et al.* (1996)
D	SAD	Retinol acetate (0.003–30 µM)	Mitomycin C (0.3 µg/mL)	TA1535/ pSK1002	–	–	NA	Okai *et al.* (1996)
D	SAD	Retinoic acid (0.003–30 µM)	Mitomycin C (0.3 µg/mL)	TA1535/ pSK1002	–	–	NA	Okai *et al.* (1996)
G	SA4	Retinol (0.1–1 µmol/plate)	Hydrogen peroxide (5 µmol/plate)	TA104	–	–	NA	Han (1992)
G	SA4	Retinoic acid (1–10 µmol/plate)	Hydrogen peroxide (5 µmol/plate)	TA104	–	–	NA	Han (1992)
G	SA2	Retinyl acetate (0.2–1 µmol/plate)	Cysteine (20 µmol/plate)	TA102	–	+	1 µmol/plate (ID50)	Stark *et al.* (1994)
G	SA9	Retinol (10–25 µg/plate)	Adriamycin (5–15 µg/plate)	TA98	–	–	NA	Baird & Birnbaum (1979)
G	SA0	Retinyl palmitate (0.33–10 µmol/plate)	4-Nitroquinoline 1–oxide (2 nmol/plate)	TA100	–	–	NA	Camoirano *et al.* (1994)

Table 26 (contd)

End-point [a]	Code [a]	Modulator (tested doses) [b]	Mutagen (tested doses) [b]	*S. typhimurium* strain	S9 mix	Result [c]	Inhibitory dose (IDx) [d]	Reference
G	SA0	Retinoic acid (0.33–10 µmol/plate)	4-Nitroquinoline 1-oxide (2 nmol/plate)	TA100	–	–	NA	Camoirano *et al.* (1994)
G	SA2	Retinol (2.5–40 µg/plate)	Mitomycin C (0.4 µg/plate)	TA102	–	–	NA	Qin & Huang (1985)
G	SA2	Retinol (2.5–40 µg/plate)	Mitomycin C (0.4 µg/plate)	TA102	+	–	NA	Qin & Huang (1985)
G	SA5	Retinol (8–16 µg/plate)	Diepoxybutane (10–50 µg/plate)	TA1535	–	–	NA	Busk & Ahlborg (1980)
G	SA8	Retinol (1–100 µM)	Photoactivated 2-azido-9-fluorenone oxime (0.1 µM)	TA1538	–	–	NA	White & Rock (1981)
G	SA9	Retinol (5–500 µg/plate)	4-Nitro-*o*-phenylendiamine (5–25 µg/plate)	TA98	–	–	NA	Balbinder *et al.* (1983)
G	SA0	Retinoic acid (0.1–0.5 µmol/plate)	MNNG (13.6 nmol/plate)	TA100	–	+	0.35 µmol/plate (ID50)	Shetty *et al.* (1988)
G	SA0	Retinyl acetate (0.1–0.5 µmol/plate)	MNNG (13.6 nmol/plate)	TA100	–	+	NA	Shetty *et al.* (1988)
G	SA0	Retinol (0.1–0.5 µmol/plate)	MNNG (13.6 nmol/plate)	TA100	–	+	0.35 µmol/plate (ID50)	Shetty *et al.* (1988)
G	SA0	Retinal (0.1–0.5 µmol/plate)	MNNG (13.6 nmol/plate)	TA100	–	+	0.14 µmol/plate (ID50)	Shetty *et al.* (1988)
G	SA0	Retinyl palmitate (0.1–0.5 µmol/plate)	MNNG (13.6 nmol/plate)	TA100	–	–	NA	Shetty *et al.* (1988)
G	SA9	Retinol (up to 1 µmol/plate)	2-Nitrofluorene (unspecified dose)	TA98	–	(+)	1 µmol/plate (ID43)	Tang & Edenharder (1997)
G	SA9	Retinal (up to 2 µmol/plate)	2-Nitrofluorene (unspecified dose)	TA98	–	+	0.2 µmol/plate (ID50)	Tang & Edenharder (1997)
G	SA9	Retinoic acid (up to 1 µmol/plate)	2-Nitropyrene (unspecified dose)	TA98	–	(+)	1 µmol/plate (ID33)	Tang & Edenharder (1997)

Table 26 (contd)

End-point [a]	Code [a]	Modulator (tested doses) [b]	Mutagen (tested doses) [b]	*S. typhimurium* strain	S9 mix	Result [c]	Inhibitory dose (IDx) [d]	Reference
G	SA9	Retinyl palmitate (up to 2 µmol/plate)	2-Nitropyrene (unspecified dose)	TA98	–	+	0.3 µmol/plate (ID50)	Tang & Edenharder (1997)
G	SA9	Retinoic acid (up to 1 µmol/plate)	1-Nitropyrene (unspecified dose)	TA98	–	(+)	1 µmol/plate (ID45)	Tang & Edenharder (1997)
G	SA9	Retinoic acid (up to 2 µmol/plate)	1-Nitropyrene (unspecified dose)	TA98	–	+	0.06 µmol/plate (ID50)	Tang & Edenharder (1997)
G	SA9	Retinoic acid (up to 1 µmol/plate)	1-Nitropyrene (unspecified dose)	TA98	–	+	1 µmol/plate (ID50)	Tang & Edenharder (1997)
G	SA9	Retinyl palmitate (up to 2 µmol/plate)	1-Nitropyrene (unspecified dose)	TA98	–	+	0.06 µmol/plate (ID50)	Tang & Edenharder (1997)
G	SA9	Retinol (up to 1 µmol/plate)	3-Nitrofluoranthene (unspecified dose)	TA98	–	+	0.1 µmol/plate (ID50)	Tang & Edenharder (1997)
G	SA9	Retinal (up to 2 µmol/plate)	3-Nitrofluoranthene (unspecified dose)	TA98	–	+	0.05 µmol/plate (ID50)	Tang & Edenharder (1997)
G	SA9	Retinoic acid (up to 1 µmol/plate)	3-Nitrofluoranthene (unspecified dose)	TA98	–	+	0.6 µmol/plate (ID50)	Tang & Edenharder (1997)
G	SA9	Retinyl palmitate (up to 2 µmol/plate)	3-Nitrofluoranthene (unspecified dose)	TA98	–	+	0.07 µmol/plate (ID50)	Tang & Edenharder (1997)
G	SA0	Retinol (1–150 µg/plate)	Methylazoxymethanol (200 µg/plate)	TA100	–	+	5 µg/plate (ID62)	Tavan *et al.* (1997)
G	SA9	Retinol (0.21–1.72 µmol/plate)	Coal dust extract (75 mg/plate)	TA98	–	(+)	1.72 µmol/plate (ID29)	Ong *et al.* (1989)
G	SA9	Retinol (0.21–1.72 µmol/plate)	Diesel emission particles extract (2 mg/plate)	TA98	–	(+)	1.72 µmol/plate (ID31)	Ong *et al.* (1989)
G	SA9	Retinol (0.21–1.72 µmol/plate)	Tobacco snuff extract (85 mg/plate)	TA98	–	(+)	1.72 µmol/plate (ID29)	Ong *et al.* (1989)

Table 26 (contd)

End-point [a]	Code [a]	Modulator (tested doses) [b]	Mutagen (tested doses) [b]	*S. typhimurium* strain	S9 mix	Result [c]	Inhibitory dose (IDx) [d]	Reference
G	SA9	Retinol (0.21–1.72 µmol/plate)	Airborne particles extract (4 mg/plate)	TA98	–	(+)	3.45 µmol/plate (ID45)	Ong *et al.* (1989)
G	SA9	Retinol (0.21–1.72 µmol/plate)	Fried beef extract (750 mg/plate)	TA98	+	(+)	1.72 µmol/plate (ID48)	Ong *et al.* (1989)
G	SA9	Retinol (1.6–16 µg/plate)	Aflatoxin B_1 (0.04 µg/plate)	TA98	+	+	16 µg/plate (ID70)	Busk & Ahlborg (1980)
G	SA9	Retinol (0.0002–2 µM)	Aflatoxin B_1 (0.2 µg/plate)	TA98	+	+	0.0006 µM (ID55)	Raina & Gurtoo (1985)
G	SA0	Retinol (0.0002–2 µM)	Aflatoxin B_1 (0.2 µg/plate)	TA100	+	+	0.0002 µM (ID70)	Raina & Gurtoo (1985)
G	SA9	Retinoic acid (0.0002–2 µM)	Aflatoxin B_1 (0.2 µg/plate)	TA98	+	+	0.0002 µM (ID55)	Raina & Gurtoo (1985)
G	SA0	Retinoic acid (0.0002–2 µM)	Aflatoxin B_1 (0.2 µg/plate)	TA100	+	–	NA	Raina & Gurtoo (1985)
G	SA9	Retinol (2.5–40 µg/plate)	Aflatoxin B_1 (0.5 µg/plate)	TA98	+	+	10 µg/plate (ID50)	Qin & Huang (1985)
G	SA9	Retinoic acid (2.5–40 µg/plate)	Aflatoxin B_1 (0.5 µg/plate)	TA98	+	+	10 µg/plate (ID50)	Qin & Huang (1985)
G	SA0	Retinal (0.1–0.5 µmol/plate)	Aflatoxin B_1 (1.28 nmol/plate)	TA100	+	+	0.1 µmol/plate (ID90)	Bhattarcharya *et al.* (1987)
G	SA0	Retinoic acid (0.1–0.5 µmol/plate)	Aflatoxin B_1 (1.28 nmol/plate)	TA100	+	+	0.1 µmol/plate (ID66)	Bhattarcharya *et al.* (1987)
G	SA0	Retinyl acetate (0.1–0.5 µmol/plate)	Aflatoxin B_1 (1.28 nmol/plate)	TA100	+	+	0.1 µmol/plate (ID50)	Bhattarcharya *et al.* (1987)
G	SA0	Retinyl palmitate (0.1–0.5 µmol/plate)	Aflatoxin B_1 (1.28 nmol/plate)	TA100	+	+	0.5 µmol/plate (ID50)	Bhattarcharya *et al.* (1987)

Table 26 (contd)

End-point [a]	Code [a]	Modulator (tested doses) [b]	Mutagen (tested doses) [b]	*S. typhimurium* strain	S9 mix	Result [c]	Inhibitory dose (IDx) [d]	Reference
G	SA9	Retinyl acetate [No direct treatment – S9 from mice maintained on a vitamin A-deficient diet or on a retinyl acetate-supplemented diet (20 µg/g diet) for 10 weeks]	Aflatoxin B_1 (0.1–0.4 µg/plate)	TA98	+	(+)	NA (ID24)	Qin & Huang (1986)
S	SA9	Retinyl palmitate [no direct treatment – S9 from rats maintained on a retinyl palmitate-normal diet (5 IU/g diet) or a retinyl palmitate-supplemented diet (500 IU/g diet) for 8 days]	Aflatoxin B_1 (2.5–100 ng/plate)	TA98	+	+	NA (ID78)	Decoudu *et al.* (1992)
G	SA9	Retinol (27.5-136 µM)	BP (2.75 µM)	TA98	+	+	27.5 µM (ID40)	Calle & Sullivan (1982)
G	SA9	Retinol (2.5–40 µg/plate)	BP (5 µg/plate)	TA98	+	–	NA	Qin & Huang (1985)
G	SA9	Retinoic acid (2.5–40 µg/plate)	BP (5 µg/plate)	TA98	+	–	NA	Qin & Huang (1985)
G	SA9	Retinyl acetate (2.5–40 µg/plate)	BP (5 µg/plate)	TA98	+	–	NA	Qin & Huang (1985)
G	SA9	Retinyl acetate [No direct treatment – S9 from mice maintained on a vitamin A-deficient diet or on a retinyl acetate-supplemented diet (20 µg/g diet) for 10 weeks]	BP (25–100 µg/plate)	TA98	+	–	NA	Qin & Huang (1986)
G	SA9	Retinyl acetate (80–800 µg/plate)	BP (10 µg/plate)	TA98	+	(+)	800 µg/plate (ID43)	Balansky *et al.* (1994)
S	SA9	Retinyl palmitate (40–4000 µg/plate)	BP (1–1000 µg/plate)	TA98	+	+	400 µg/plate (ID64 with 1 µg BP)	Alzieu *et al.* (1987)

Table 26 (contd)

End-point [a]	Code [a]	Modulator (tested doses) [b]	Mutagen (tested doses) [b]	*S. typhimurium* strain	S9 mix	Result [c]	Inhibitory dose (IDx) [d]	Reference
S	SA9	Retinyl palmitate [No direct treatment – S9 from rats maintained on a retinyl acetate-deficient diet or on a retinyl palmitate diet (20 IU/g diet) for 8 weeks]	BP (50 µg/plate)	TA98	+	(+)	NA (ID37)	Colin *et al.* (1991)
S	SA9	Retinyl palmitate [No direct treatment – S9 from rats maintained on a retinyl acetate-deficient diet or S9 from rats receiving an i.p. injection of retinyl palmitate (750 mg/kg bw) 48 h before sacrifice]	BP (50 µg/plate)	TA98	+	(+)	NA (ID22)	Colin *et al.* (1991)
G	SA9	Retinol (2.8–140 nmol/plate)	6-Methylbenzo[*a*]pyrene (2.8 nmol/plate)	TA98	+	(+)	140 nmol/plate (ID50)	Bayless *et al.* (1986)
G	SA9	Retinol (1.3–65 nmol/plate)	6-Hydroxymethylbenzo-[*a*]pyrene (1.3 nmol/plate)	TA98	+	(+)	26 nmol/plate (ID50)	Bayless *et al.* (1986)
G	SA9	Retinol (0.6–30 nmol/plate)	6–Acetoxymethylbenzo-[*a*]pyrene (0.6 nmol/plate)	TA98	–	–	NA	Bayless *et al.* (1986)
G	SA0	Retinol (2.5–40 µg/plate)	Benz[*a*]anthracene (5 µg/plate)	TA100	+	–	NA	Qin & Huang (1985)
G	SA0	Retinol (2.5–40 µg/plate)	DMBA (20 µg/plate)	TA100	+	–	NA	Qin & Huang (1985)
G	SA0	Retinol (2.5–40 µg/plate)	MCA (5 µg/plate)	TA100	+	+	20 µg/plate (ID50)	Qin & Huang (1985)
G	SA9	Retinol (5–100 µg/plate)	2-Aminofluorene (0.25–1 µg/plate)	TA98	+	+	10 µg/plate (ID100)	Baird & Birnbaum (1979)

Table 26 (contd)

End-point [a]	Code [a]	Modulator (tested doses) [b]	Mutagen (tested doses) [b]	*S. typhimurium* strain	S9 mix	Result [c]	Inhibitory dose (IDx) [d]	Reference
G	SA9	Retinol (2–100 µg/plate)	2-Aminofluorene (0.3–0.6 µg/plate)	TA98	+	§	NA	Busk & Ahlborg (1982a)
G	SA9	Retinol (2–100 µg/plate)	2-Acetylaminofluorene (2 µg/plate)	TA98	+	§	NA	Busk & Ahlborg (1982a)
G	SA9	Retinol (5–500 µg/plate)	2-Aminofluorene (2.5–10 µg/plate)	TA98	+	§	NA	Balbinder *et al.* (1983)
G	SA9	Retinol (2.5–150 µg/plate)	*N*-Hydroxy-2-acetyl-aminofluorene (50 µg/plate)	TA98	+	#	NA	Rondahl *et al.* (1985)
G	SA9	Retinol (2.5–150 µg/plate)	*N*-Hydroxy-2-acetyl-aminofluorene (50 µg/plate)	TA98	-	-	NA	Rondahl *et al.* (1985)
G	SA0	Retinol (0.017–0.525 µmol/plate)	*o*-Aminoazotoluene (5 µg/plate)	TA100	+	+	0.07 µmol/plate (ID50)	Busk & Ahlborg (1982b)
G	SA0	Retinyl acetate (0.017–0.525 µmol/plate)	*o*-Aminoazotoluene (5 µg/plate)	TA100	+	+	0.07 µmol/plate (ID50)	Busk & Ahlborg (1982b)
G	SA0	Retinyl palmitate (0.017–0.175 µmol/plate)	*o*-Aminoazotoluene (5 µg/plate)	TA100	+	(+)	0.175 µmol/plate (ID30)	Busk & Ahlborg (1982b)
G	SA0	Retinol (2–150 µg/plate)	*o*-Aminoazotoluene (7 µg/plate)	TA100	+ (Gerbil)	+	10 µg/plate (ID50)	Victorin *et al.* (1987)
G	SA0	Retinol (2–150 µg/plate)	*o*-Aminoazotoluene (15 µg/plate)	TA100	+ (Mouse)	+	50 µg/plate (ID50)	Victorin *et al.* (1987)
G	SA0	Retinol (2–150 µg/plate)	*o*-Aminoazotoluene (10 µg/plate)	TA100	+ (Hamster)	+	50 µg/plate (ID50)	Victorin *et al.* (1987)
G	SA0	Retinol (2–150 µg/plate)	*o*-Aminoazotoluene (8 µg/plate)	TA100	+ (Rat)	+	150 µg/plate (ID50)	Victorin *et al.* (1987)
G	SA5	Retinol (0.004–0.21 µmol/plate)	Cyclophosphamide (400 µg/plate)	TA1535	+	+	0.105 µmol/plate (ID56)	Busk *et al.* (1984)
G	SA5	Retinyl acetate (0.004–0.21 µmol/plate)	Cyclophosphamide (400 µg/plate)	TA1535	+	+	0.105 µmol/plate (ID70)	Busk *et al.* (1984)

Table 26 (contd)

End-point [a]	Code [a]	Modulator (tested doses) [b]	Mutagen (tested doses) [b]	*S. typhimurium* strain	S9 mix	Result [c]	Inhibitory dose (IDx) [d]	Reference
G	SA5	Retinyl palmitate (0.004–0.21 µmol/plate)	Cyclophosphamide (400 µg/plate)	TA1535	+	(+)	0.21 µmol/plate (ID40)	Busk *et al.* (1984)
G	SA5	Retinol (2.5–40 µg/plate)	Cyclophosphamide (200 µg/plate)	TA1535	+	+	10 µg/plate (ID50)	Qin & Huang (1985)
G	SA9	Retinol (2–150 µg/plate)	Glu–P–1 (0.0075–0.015 µg/plate)	TA98	+	+	25 µg/plate (ID50)	Busk *et al.* (1982)
G	SA9	Retinol (2–150 µg/plate)	Glu–P–2 (0.5–1 µg/plate)	TA98	+	+	20 µg/plate (ID50)	Busk *et al.* (1982)
G	SA9	Retinol (2–150 µg/plate)	Trp-P-1 (0.04–0.08 µg/plate)	TA98	+	+	20 µg/plate (ID50)	Busk *et al.* (1982)
G	SA9	Retinol (2–150 µg/plate)	Trp-P-2 (0.005–0.01 µg/plate)	TA98	+	+	5 µg/plate (ID50)	Busk *et al.* (1982)
G	SA9	Retinol (25–100 µg/plate)	IQ (0.025–0.1 µg/plate)	TA98	+	+	50 µg/plate (ID50)	Ioannides *et al.* (1990)
G	SA9	Retinal (25–100 µg/plate)	IQ (0.1 µg/plate)	TA98	+	(+)	100 µg/plate (ID50)	Ioannides *et al.* (1990)
G	SA9	Retinol (25–100 µg/plate)	MeIQ (0.1 µg/plate)	TA98	+	(+)	100 µg/plate (ID28)	Ioannides *et al.* (1990)
G	SA9	Retinal (25–100 µg/plate)	MeIQ (0.1 µg/plate)	TA98	+	(+)	75 µg/plate (ID50)	Ioannides *et al.* (1990)
G	SA9	Retinol (25–100 µg/plate)	MeIQx (0.1 µg/plate)	TA98	+	+	50 µg/plate (ID50)	Ioannides *et al.* (1990)

Table 26 (contd)

End–point [a]	Code [a]	Modulator (tested doses) [b]	Mutagen (tested doses) [b]	*S. typhimurium* strain	S9 mix	Result [c]	Inhibitory dose (IDx) [d]	Reference
G	SA9	Retinal (25–100 µg/plate)	MeIQx (0.1 µg/plate)	TA98	+	(+)	100 µg/plate (ID50)	Ioannides *et al.* (1990)
G	SA0	Retinol (5–40 µg/plate)	*N*-Nitrosodimethylamine (100 µg/plate)	TA100	+	+	40 µg/plate (ID90)	Huang (1987)
G	SA0	Retinol (5–40 µg/plate)	*N*-Nitrosodiethylamine (100 µg/plate)	TA100	+	+	40 µg/plate (ID60)	Huang (1987)
G	SA9	Retinol acetate (80–800 µg/plate)	Cigarette smoke (43 mL/Lair for 1–5 min)	TA98	+	+	400 µg/plate (ID60)	Balansky *et al.* (1994)
G	SA9	Retinyl palmitate (1–10 µmol/plate)	Cigarette smoke (18 mL/L air for 6 min)	TA98	+	–	NA	Camoirano *et al.* (1994)
G	SA9	Retinoic acid (1–10 µmol/plate)	Cigarette smoke (18 mL/L air for 6 min)	TA98	+	–	NA	Camoirano *et al.* (1994)
G	SA9	Retinol (0.025–0.5 µmol/plate)	Cigarette smoke condensate (100–400 µg/plate)	TA98	+	–	NA	Wilmer & Spit (1986)
G	SA9	Retinoic acid (0.025–0.5 µmol/plate)	Cigarette smoke condensate (100–400 µg/plate)	TA98	+	–	NA	Wilmer & Spit (1986)
G	SA9	Retinyl acetate (0.025–0.5 µmol/plate)	Cigarette smoke condensate (100–400 µg/plate)	TA98	+	–	NA	Wilmer & Spit (1986)
G	SA9	Retinol (50–800 µg/plate)	Cigarette smoke condensate (400 µg/plate)	TA98	+	+	400 µg/plate (ID50)	Romert *et al.* (1994)
G	SA0	Retinol (50–800 µg/plate)	Cigarette smoke condensate (400 µg/plate)	TA100	+	+	100 µg/plate (ID50)	Romert *et al.* (1994)

[a] End-points investigated are: D, DNA damage; G, gene mutation; test codes are given in Appendix 2

[b] Doses of compounds are as reported by the authors

[c] +, inhibition of genotoxicity (> ID50); (+), weak inhibition of genotoxicity (< ID50); –, no inhibition of genotoxicity; #, increase of genotoxicity; §, variable effect, depending on the dose of retinol and on the amount of S9 fraction per plate

[d] Inhibitory dose (IDx), dose inhibiting the genotoxicity by x%, as indicated by the authors or inferred from their data; NA, not applicable.

Abbreviations: BP, benzo[*a*]pyrene; DMBA, 7,12-dimethylbenz[*a*]anthracene; MNU, *N*-methyl-*N*-nitrosourea; MCA, 3-methylcholanthrene; IQ, 2-amino-3-methyl-imidazo[4,5-*f*]quinoline; MeIQ, 2-amino-3,4-dimethylimidazo[4,5-*f*]quinoline; MeIQx, 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline; Glu-P-1, 2-amino-6-methylpyrido[1,2-*a*:3',2'-*d*]imidazole; Glu-P-2, 2-aminodipyrido[1,2-*a*:3',2'-*d*]imidazole; Trp-P-1, 3-amino-3,4-dimethyl-5*H*-pyrido[4,3-*b*]indole; Trp-P-2, 3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole; MNNG, *N*-methyl-*N*'nitro-*N*-nitrosoguanidine; NNK, 4-(*N*-nitrosomethylamino)-1-(3-pyridyl)-1-butanone

A-deficient diet or a retinyl acetate-supplemented diet were used (Qin & Huang, 1986). On the other hand, higher doses of retinol (Calle & Sullivan, 1982), retinyl acetate (Balansky *et al.*, 1994) and retinyl palmitate (Alzieu *et al.*, 1987) inhibited the mutagenicity of BP in TA98. Moreover, the mutagenic potency of BP, in the presence of liver S9 fractions from Sprague–Dawley rats maintained on a diet lacking retinyl acetate, was higher than in the presence of S9 either from rats maintained for eight weeks on a retinyl acetate-sufficient diet or from rats receiving an intraperitoneal injection of retinyl acetate 48 h before sacrifice (Colin *et al.*, 1991). Retinol was weakly antimutagenic towards two S9-requiring derivatives of BP (6-methylbenzo[*a*]pyrene and 6-hydroxymethylbenzo[*a*]pyrene), whereas it did not affect the mutagenicity of 6-acetoxymethylbenzo[*a*]pyrene (Bayless *et al.*, 1986).

Retinol and retinyl acetate inhibited the mutagenicity of 2-aminofluorene in TA98 following metabolic activation by either rat liver microsomes or S9 fractions (Baird & Birnbaum, 1979). Other studies showed variable effects of retinol on the mutagenicity of 2-aminofluorene and 2-acetylaminofluorene in TA98. In fact, low doses of retinol even increased the mutagenicity of both aromatic amines, whereas high doses did not affect the mutagenicity of 2-acetylaminofluorene and decreased the mutagenicity of 2-aminofluorene (Busk & Ahlborg, 1982a). A similar diphasic effect was observed in testing retinol towards 2-aminofluorene in another laboratory (Balbinder *et al.*, 1983). Retinol also enhanced the mutagenicity of *N*-hydroxy-2-acetylaminofluorene (Rondahl *et al.*, 1985).

Retinol and retinyl acetate and, less effectively, retinyl palmitate decreased the mutagenicity of *o*-aminoazotoluene in TA100 (Busk & Ahlborg, 1982b) and of cyclophosphamide in TA1535 (Busk *et al.*, 1984; Qin & Huang, 1985). In a further study by the same group (Victorin *et al.*, 1987), inhibition of of the activity of *o*-aminoazotoluene by retinol was confirmed when this azo-dye was activated with liver S9 fractions from four rodent species (gerbil, mouse, hamster and rat).

Two studies have demonstrated that retinol and retinal decrease the mutagenicity in TA98 of heterocyclic amines isolated from food pyrolysates. In particular, retinol decreased the mutagenicity of 2-amino-6-methyldipyrido-[1,2-*a*:3′,2′-*d*]imidazole (Glu-P-1), 2-aminodipyrido[1,2-*a*:3′,2′-*d*]imidazole (Glu-P-2) and 3-amino-1,4-dimethyl-5*H*-pyrido[4,3-*b*]indole (Trp-P-1) and, with higher efficiency, the mutagenicity of 3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole (Trp-P-2) (Busk *et al.*, 1982). Both retinol and retinal, when incorporated into the rat liver S9 activation system, caused a dose-related decrease of the mutagenicity of the aminoimidazoazaarenes 2-amino-3-methylimidazo[4,5-*f*]-quinoline (IQ), 2-amino-3,4-dimethylimidazo[4,5-*f*]quinoline (MeIQ) and 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx). Several parallel findings led to the conclusion that retinol lowers IQ mutagenicity through inhibition of its cytochrome P450-dependent metabolic activation (Ioannides *et al.*, 1990).

Retinol weakly inhibits the S9-mediated mutagenicity of both *N*-nitrosodimethylamine and *N*-nitrosodiethylamine in TA100 in the presence of mouse liver S9 fractions (Huang, 1987).

Assays of the S9-mediated mutagenicity of cigarette smoke and condensates from it have given conflicting results. In one study, retinol, retinoic acid and retinyl acetate did not affect the mutagenicity of a cigarette smoke condensate in TA98 (Wilmer & Spit, 1986). In contrast, in another laboratory, retinol exerted protective effects in both TA98 and TA100, although the authors noted that occurrence of toxic effects could not be excluded (Romert *et al.*, 1994). The mutagenicity of mainstream cigarette smoke in TA98 was inhibited by retinyl acetate (Balansky *et al.*, 1994) but was unaffected by either retinyl palmitate or retinoic acid (Camoirano *et al.*, 1994).

In the 1–10 µg/mL range, retinol attenuated the differential toxicity of *N*-nitrosodimethylamine and *N*-nitrosopyrrolidine in the *Escherichia coli* strains K-12 343/765 (*uvr*$^+$/*rec*$^+$) and K-12 343/753 (*uvrB*$^-$/*recA*$^-$). In a host-mediated assay in Swiss albino mice, administration of retinol by gavage (250 mg/kg bw) inhibited the differential toxicity in these two *E. coli* strains. Bacteria were injected intravenously and recovered from blood, lungs, liver, spleen,

kidneys and testes of mice treated by gavage with *N*-nitrosodimethylamine (80 mg/kg bw) or from liver and spleen of mice treated by gavage with *N*-nitrosopyrrolidine (Knasmüller *et al.*, 1992).

As summarized in Table 27, retinol, retinal, retinoic acid and retinyl esters have been investigated for their ability to influence a variety of genetic and related end-points in mammalian cultured cells. Retinol inhibited the induction of DNA single-strand breaks induced by the nicotine-derived nitrosamine NNK in primary rat hepatocytes (Alaoui-Jamali *et al.*, 1991a). Retinol and retinoic acid did not affect the unscheduled DNA synthesis (UDS) induced by either ethyl methanesulfonate or 254-nm ultraviolet light in primary rat hepatocytes, but were effective in attenuating the UDS induced by DMBA, which requires activation to genotoxic metabolites (Budroe *et al.*, 1987). Retinol increased BP-induced UDS in hamster tracheal epithelium in organ culture. In the same system, retinol did not significantly affect the levels of BP diol epoxide–DNA adducts, as measured by ^{32}P-postlabelling, but reduced the formation of DNA adducts as detected by immunocytochemistry. The authors suggested that, like β-carotene, retinol may protect the respiratory epithelium by enhancing DNA repair activity and removing DNA adducts (Wolterbeek *et al.*, 1995). Retinol decreased the formation of DNA adducts in cultured woodchuck hepatocytes treated with [^{3}H]aflatoxin B_1 (Yu *et al.*, 1994).

Retinol and retinoic acid inhibited the induction of mutations at the HGPRT locus in Chinese hamster ovary cells when DMBA was used as a mutagenic agent, in the presence of an exogenous metabolic activation system (rat liver S9), but not when the direct-acting mutagen ethyl methanesulfonate was used (Budroe *et al.*, 1988).

The protective effects of vitamin A have been extensively investigated in terms of the frequency of sister chromatid exchanges (SCE) in Chinese hamster cells. In V79 cells, no effect was produced on the SCE induced by direct-acting mutagens, including mitomycin C, in the presence of retinol, retinal, retinoic acid and retinyl acetate (Sirianni *et al.*, 1981), or by MNNG and ethyl methanesulfonate in the presence of retinol (Qin *et al.*, 1985). However, retinol significantly inhibited the metabolic deactivation of MNNG in the presence of S9 (Qin *et al.*, 1985). With the exception of the polycyclic aromatic hydrocarbons benz[*a*]anthracene, BP, DMBA and MCA, whose SCE-inducing activity was not affected by retinol (Qin *et al.*, 1985), retinol was protective towards other genotoxic agents requiring metabolic activation. These included cyclophosphamide and aflatoxin B_1 (Sirianni *et al.*, 1981; Qin *et al.*, 1985), *N*-nitrosodimethylamine and *N*-nitrosodiethylamine (Huang, 1987), in the presence of S9 mix, and NNK in primary rat hepatocytes (Alaoui-Jamali *et al.*, 1991a). A delay in the cell cycle was also observed (Huang, 1987). In Chinese hamster epithelial liver cells, which retain the intrinsic capacity to metabolize carcinogens, retinol and retinoic acid weakly inhibited the SCE-inducing activity of cyclophosphamide and DMBA (Cozzi *et al.*, 1990).

Chromosomal aberrations in Chinese hamster V79 cells, in the presence of S9 mix, were significantly inhibited when aflatoxin B_1 and cyclophosphamide were used as clastogenic agents. In contrast, they were significantly enhanced when BP and DMBA were used (Qin *et al.*, 1985).

The morphological transformation of C3H 10T1/2 mouse fibroblasts, pre-treated with MCA seven days earlier, was inhibited by retinol, retinal and retinyl acetate with similar potency. This effect was reversed when cell cultures were further maintained in a retinoid-free medium (Merriman & Bertram, 1979).

In human cultured cells (Table 28), preincubation with retinoic acid for two days did not affect the formation of DNA adducts in endometrioid $IGROV_1$ cells, but enhanced their formation in ovarian carcinoma NIHOVCAR cells at various times (0, 24 or 48 h) after treatment with cisplatin. This effect was accompanied by potentiation of cisplatin toxicity (Caliaro *et al.*, 1997). The mutagenic activity of polycyclic aromatic hydrocarbons, as evaluated by selection against diphtheria toxin, was decreased by vitamin A in human heteroploid epithelial-like (EUE) cells. In particular, retinyl palmitate protected these cells against the mutagenicity of

Table 27. Assay of retinol, retinal, retinoic acid and retinyl esters for ability to inhibit genetic and related effects in cultured animal cells

End-point[a]	Code[a]	Modulator (tested doses)[b]	Genotoxic agent (tested doses)[b]	Cells	Investigated effect	Result[c]	Inhibitory dose (IDx)[d]	Reference
D	DIA	Retinol (69.8 µM)	NNK (1–10 mM)	Primary rat hepatocytes	DNA single-strand breaks	(+)	69.8 µM (ID13)	Alaoui-Jamali *et al.* (1991a)
D	URP	Retinol (1–50 µM)	Ethyl methanesulfonate (200 µg/mL) [1.6 mM]	Primary rat hepatocytes	Unscheduled DNA synthesis	–	NA	Budroe *et al.* (1987)
D	URP	Retinoic acid (1–50 µM)	Ethyl methanesulfonate (200 µg/mL) [1.6 mM]	Primary rat hepatocytes	Unscheduled DNA synthesis	–	NA	Budroe *et al.* (1987)
D	URP	Retinol (1–50 µM)	254 nm UV light (32 J/m^3)	Primary rat hepatocytes	Unscheduled DNA synthesis	–	NA	Budroe *et al.* (1987)
D	URP	Retinoic acid (1–50 µM)	254 nm UV light (32 J/m^3)	Primary rat hepatocytes	Unscheduled DNA synthesis	–	NA	Budroe *et al.* (1987)
D	URP	Retinol (1–50 µM)	DMBA (5 µg/mL) [20 µM]	Primary rat hepatocytes	Unscheduled DNA synthesis	+	1 µM (ID55)	Budroe *et al.* (1987)
D	URP	Retinoic acid (1–50 µM)	DMBA (5 µg/mL) [20 µM]	Primary rat hepatocytes	Unscheduled DNA synthesis	+	1 µM (ID65)	Budroe *et al.* (1987)
D	UIA	Retinol (10 µM)	BP (10 µM)	Hamster tracheal epithelium in organ culture	Unscheduled DNA synthesis	#	NA	Wolterbeek *et al.* (1995)
D	BID	Retinol (10 µM)	BP (10 µM)	Hamster tracheal epithelium in organ culture	BPDE–DNA adducts	(+)	10 µM (ID20)	Wolterbeek *et al.* (1995)
D	BID	Retinol (34–122 µM)	Aflatoxin B_1 (0.08–10 µM)	Woodchuck hepatocytes	DNA adducts	+	34 µM (ID50)	Yu *et al.*, (1994)
G	GCO	Retinol (1–25 µM)	Ethyl methanesulfonate (100 µg/mL) [810 µM]	Chinese hamster ovary (CHO) cells	Mutation at the HGPRT locus	–	NA	Budroe *et al.* (1988)
G	GCO	Retinoic acid (1–25 µM)	Ethyl methanesulfonate (100 µg/mL) [810 µM]	Chinese hamster ovary (CHO) cells	Mutation at the HGPRT locus	–	NA	Budroe *et al.* (1988)

Table 27 (contd)

End-point[a]	Code[a]	Modulator (tested doses)[b]	Genotoxic agent (tested doses)[b]	Cells	Investigated effect	Result[c]	Inhibitory dose (IDx)[d]	Reference
G	GCO	Retinol (1–25 µM)	DMBA (5 µg/mL) [20 µM]	Chinese hamster ovary (CHO) cells + S9	Mutation at the HGPRT locus	+	5 µM (ID63)	Budroe *et al.* (1988)
G	GCO	Retinoic acid (1–25 µM)	DMBA (5 µg/mL) [20 µM]	Chinese hamster ovary (CHO) cells + S9	Mutation at the HGPRT locus	+	5 µM (ID60)	Budroe *et al.* (1988)
G	G9H	Retinyl acetate (100 µg/mL)	Ethyl methanesulfonate (100–1000 µg/mL)	Chinese hamster V79 cells	6-TG-resistance mutation	+	100 µg/mL (ID65)	Kuroda (1990)
S	SIC	Retinol (1–4 µg/mL)	Mitomycin C (0.03 µg/mL)	Chinese hamster V79 cells	Sister chromatid exchanges	–	NA	Sirianni *et al.* (1981)
S	SIC	Retinal (0.25–1 µg/mL)	Mitomycin C (0.03 µg/mL)	Chinese hamster V79 cells	Sister chromatid exchanges	–	NA	Sirianni *et al.* (1981)
S	SIC	Retinoic acid (1–4 µg/mL)	Mitomycin C (0.03 µg/mL)	Chinese hamster V79 cells	Sister chromatid exchanges	–	NA	Sirianni *et al.* (1981)
S	SIC	Retinyl acetate (1–4 µg/mL)	Mitomycin C (0.03 µg/mL)	Chinese hamster V79 cells	Sister chromatid exchanges	–	NA	Sirianni *et al.* (1981)
S	SIC	Retinol (1–8 µg/mL)	Cyclophosphamide (5 µg/mL)	Chinese hamster V79 cells + S9	Sister chromatid exchanges	+	8 µg/mL (ID65)	Huang *et al.* (1982)
S	SIC	Retinol (1–64 µg/mL)	Aflatoxin B_1 (0.2–0.5 µg/mL)	Chinese hamster V79 cells + S9	Sister chromatid exchanges	(+)	32 µg/mL (ID40)	Huang *et al.* (1982)
S	SIC	Retinol (4–32 µg/mL)	*N*-Nitrosodimethylamine (0.5 mg/mL)	Chinese hamster V79 cells + S9	Sister chromatid exchanges	+	32 µg/mL (ID55)	Huang (1987)
S	SIC	Retinol (17.4–139.6 µM)	NNK (20 mM)	Chinese hamster V79 cells + primary rat hepatocytes	Sister chromatid exchanges	+	139.6 µM (ID55)	Alaoui-Jamali *et al.* (1991a)
S	SIC	Retinol (4–32 µg/mL)	*N*-Nitrosodiethylamine (10 mg/mL)	Chinese hamster V79 cells + S9	Sister chromatid exchanges	(+)	32 µg/mL (ID44)	Huang (1987)

Table 27 (contd)

End-point[a]	Code[a]	Modulator (tested doses)[b]	Genotoxic agent (tested doses)[b]	Cells	Investigated effect	Result[c]	Inhibitory dose (IDx)[d]	Reference
S	SIC	Retinol (2–16 µg/mL)	MNNG (0.015 µg/mL)	Chinese hamster V79 cells	Sister chromatid exchanges	–	NA	Qin *et al.* (1985)
S	SIC	Retinol (2–16 µg/mL)	Ethyl methanesulfonate (250 µg/mL)	Chinese hamster V79 cells	Sister chromatid exchanges	–	NA	Qin *et al.* (1985)
S	SIC	Retinol (2–16 µg/mL)	Ethyl methanesulfonate (250 µg/mL)	Chinese hamster V79 cells + S9	Sister chromatid exchanges	–	NA	Qin *et al.* (1985)
S	SIC	Retinol (0.5–2 µg/mL)	Aflatoxin B_1 (0.2 µg/mL)	Chinese hamster V79 cells + S9	Sister chromatid exchanges	+	0.5 µg/mL (ID56)	Qin *et al.* (1985)
S	SIC	Retinol (0.5–2 µg/mL)	Cyclophosphamide (5 µg/mL)	Chinese hamster V79 cells + S9	Sister chromatid exchanges	+	0.5 µg/mL (ID56)	Qin *et al.* (1985)
S	SIC	Retinol (2–16 µg/mL)	Benz[*a*]anthracene (5 µg/mL)	Chinese hamster V79 cells + S9	Sister chromatid exchanges	–	NA	Qin *et al.* (1985)
S	SIC	Retinol (2–16 µg/mL)	Benzo[*a*]pyrene (2 µg/mL)	Chinese hamster V79 cells + S9	Sister chromatid exchanges	–	NA	Qin *et al.* (1985)
S	SIC	Retinol (2–16 µg/mL)	DMBA (2 µg/mL)	Chinese hamster V79 cells + S9	Sister chromatid exchanges	–	NA	Qin *et al.* (1985)
S	SIC	Retinol (2–16 µg/mL)	3-Methylcholanthrene (5 µg/mL)	Chinese hamster V79 cells + S9	Sister chromatid exchanges	–	NA	Qin *et al.* (1985)
S	SIC	Retinol (25 µM)	Cyclophosphamide (1 mM)	Chinese hamster epithelial liver (CHEL) cells	Sister chromatid exchanges	+	25 µM (ID50)	Cozzi *et al.* (1990)
S	SIC	Retinol (25 µM)	DMBA (0.078 µM)	Chinese hamster epithelial liver (CHEL) cells	Sister chromatid exchanges	+	25 µM (ID50)	Cozzi *et al.* (1990)
S	SIC	Retinoic acid (25–50 µM)	Cyclophosphamide (1 mM)	Chinese hamster epithelial liver (CHEL) cells	Sister chromatid exchanges	+	50 µM (ID50)	Cozzi *et al.* (1990)

Table 27 (contd)

End-point[a]	Code[a]	Modulator (tested doses)[b]	Genotoxic agent (tested doses)[b]	Cells	Investigated effect	Result[c]	Inhibitory dose (IDx)[d]	Reference
S	SIC	Retinoic acid (25–50 µM)	DMBA (0.078 µM)	Chinese hamster epithelial liver (CHEL) cells	Sister chromatid exchanges	+	37 µM (ID50)	Cozzi *et al.* (1990)
C	CIC	Retinol (4–32 µg/mL)	Aflatoxin B_1 (2 µg/mL)	Chinese hamster V79 cells + S9	Chromosomal aberrations	+	32 µg/mL (ID56)	Qin *et al.* (1985)
C	CIC	Retinol (4–32 µg/mL)	Cyclophosphamide (150 µg/mL)	Chinese hamster V79 cells + S9	Chromosomal aberrations	+	16 µg/mL (ID77)	Qin *et al.* (1985)
C	CIC	Retinol (4–32 µg/mL)	BP (100 µg/mL)	Chinese hamster V79 cells + S9	Chromosomal aberrations	#	NA	Qin *et al.* (1985)
C	CIC	Retinol (4–32 µg/mL)	DMBA (100 µg/mL)	Chinese hamster V79 cells + S9	Chromosomal aberrations	#	NA	Qin *et al.* (1985)
T	TCM	Retinol (0.05–0.5 µg/mL)	Pre-treatment with 3-methylcholanthrene (2.5 µg/mL)	C3H 10T1/2 mouse fibroblasts	Cell transformation	+	0.05 µg/mL (ID66)	Merriman & Bertram (1979)
T	TCM	Retinal (0.05–0.5 µg/mL)	Pre-treatment with 3-methylcholanthrene (2.5 µg/mL)	C3H 10T1/2 mouse fibroblasts	Cell transformation	+	0.05 µg/mL (ID75)	Merriman & Bertram (1979)
T	TCM	Retinyl acetate (0.005–0.5 µg/mL)	Pre-treatment with 3-methylcholanthrene (2.5 µg/mL)	C3H 10T1/2 mouse fibroblasts	Cell transformation	+	0.05 µg/mL (ID67)	Merriman & Bertram (1979)
T	TCM	Retinol (0.01–1 µM)	Areca nut extracts (2.5–5 mg/mL)	PdPBV-1-transfected C3H 10T1/2 mouse fibroblasts	Cell transformation	+	0.3 µM (ID100)	Stich & Tsang (1989)

[a] End-points investigated are: D, DNA damage; G, gene mutation; S, sister chromatid exchange; C, chromosomal aberration; T, cell transformation; test codes are given in Appendix 2

[b] Doses of compounds are as reported by the authors

[c] +, inhibition of the investigated effect ≥*ID50); (+), weak inhibition of the effect (≤ID50); –, no inhibition of the effect; #, enhancement of the effect; ?, unclear effect*

[d] Inhibitory dose (IDx), dose inhibiting x% of the investigated effect; NA, not applicable.

Abbreviations: BP, benzo[*a*]pyrene; DMBA, 7,12-dimethylbenz[*a*]anthracene; MNNG, *N*-methyl-*N*'nitro-*N*-nitrosoguanidine; NNK, 4-(N-nitrosomethylamino)-1-(3-pyridyl)-1-butanone

Table 28. Assay of retinol, retinal, retinoic acid and retinyl esters for ability to inhibit genetic and related effects in cultured human cells

End-point[a]	Code[a]	Modulator (tested doses)[b]	Genotoxic agent (tested doses)[b]	Cells	Investigated Effect	Result[c]	Inhibitory dose (IDx)[d]	Reference
D	BID	Retinoic acid (1 µM)	Cisplatin (10 µg/mL)	Human ovarian carcinoma NIHOVCAR cells	Pt–DNA adducts	#	NA	Caliaro *et al.* (1997)
D	BID	Retinoic acid (1 µM)	Cisplatin (10 µg/mL)	Human endometrioid $IGROV_1$ cells	Pt–DNA adducts	–	NA	Caliaro *et al.* (1997)
G	GIH	Retinyl palmitate (10 µM)	Benzo[*a*]pyrene (1 µM)	Human heteroploid epithelial-like (EUE) cells	Selection against diphtheria toxin	+	1 µM (ID87)	Rocchi *et al.* (1983)
G	GIH	Retinyl palmitate (10 µM)	3-Methylcholanthrene (1 µM)	Human heteroploid epithelial-like (EUE) cells	Selection against diphtheria toxin	+	1 µM (ID100)	Rocchi *et al.* (1983)
G	GIH	Retinyl palmitate (10 µM)	7,12-Dimethylbenz[*a*]anthracene (1 µM)	Human heteroploid epithelial-like (EUE) cells	Selection against diphtheria toxin	+	1 µM (ID63)	Rocchi *et al.* (1983)
G	GIH	Retinyl acetate (1 µM)	7,12-Dimethylbenz[*a*]anthracene (1 µM)	Human heteroploid epithelial-like (EUE) cells	Selection against diphtheria toxin	+	1 µM (ID72)	Ferreri *et al.* (1986)
G	GIH	Retinyl palmitate (10 µM)	7,12-Dimethylbenz[*a*]anthracene (1 µM)	Human heteroploid epithelial-like (EUE) cells	Selection against diphtheria toxin	+	1 µM (ID56)	Ferreri *et al.* (1986)
S	SHL	Retinol (4 µg/mL)	Melphalan (0.15 µg/mL)	Human lymphocytes	Sister chromatid exchanges	#	NA	Dozi-Vassiliades *et al.* (1985)
S	SHL	Retinol (4 µg/mL)	Caffeine (180 µg/mL)	Human lymphocytes	Sister chromatid exchanges	#	NA	Dozi-Vassiliades *et al.* (1985)

[a] End-points investigated are: D, DNA damage; G, gene mutation; S, sister chromatid exchange; test codes are given in Appendix 2

[b] Doses of compounds are as reported by the authors

[c] +, inhibition of the investigated effect *(≥ID50); (+), weak inhibition of the effect (<ID50); –, no inhibition of the effect; #, enhancement of the effect; ?, unclear effect*

[d] Inhibitory dose (IDx), dose inhibiting x% of the investigated effect; NA, not applicable.

BP, MCA and DMBA (Rocchi *et al.*, 1983). Mutagenicity of the last compound was inhibited to the same extent by retinyl acetate and retinyl palmitate (Ferreri *et al.*, 1986). Retinol even enhanced the ability of melphalan and caffeine to induce SCE in cultured human lymphocytes (Dozi-Vassiliades *et al.*, 1985).

4.3 Mechanism of chemoprevention

Mechanisms by which vitamin A might prevent or delay chemical carcinogenesis remain unclear. Various studies suggest that retinoids inhibit the promotion step of the multistage carcinogenesis process. However, there are some indications for effects on initiation as well.

4.3.1 Inhibition of events associated with carcinogenesis

Several reports described in Section 4.2.3.2 support the hypothesis that retinol and its esters inhibit the action of certain direct-acting mutagens as well as the metabolic activation of some chemical carcinogens to forms that can interact with and damage DNA. In some studies, retinol also induced the activity of detoxifying enzymes (Wang & Higuchi, 1995). These effects may be responsible, at least in part, for anticarcinogenic activity of retinol in inhibiting tumour initiation.

Evidence that retinol has protective effects against the promotion stage of carcinogenesis has come from several studies, which have demonstrated its ability to reverse premalignant lesions, inhibit cell transformation, suppress cell proliferation and DNA synthesis, and alter intercellular communication.

4.3.1.1 Effects on premalignant lesions

Administration of retinyl acetate at the time of removal of the first mammary tumour that developed after female rats had received a single injection of MNU decreased the rate of development of new tumours. Because in this carcinogenesis model the rats develop multiple asynchronous tumours, it is plausible to assume that at the time the first tumour was palpable, many preneoplastic lesions were present in the mammary gland. Therefore, these results suggest that retinyl acetate suppressed the conversion of these putative premalignant lesions to carcinomas (McCormick *et al.*, 1983). In this model, ovariectomy was also effective and the combination of retinyl acetate and ovariectomy was more effective than either agent alone. Thus, there may be a hormone-related effect of retinyl acetate. Further support for this idea comes from a study that showed that retinyl acetate inhibited estrogen-induced mammary carcinogenesis in female ACI rats, without evidence of gross toxicity (Holtzman, 1988). In this study, female rats that received a diet containing retinyl acetate (412 000 IU per kg diet) and then a subcutaneously implanted pellet containing 17α-ethinylestradiol and were maintained on retinyl acetate-containing diet for 24 further weeks developed half as many mammary cancers as rats on a control diet.

In a skin carcinogenesis model using female CD-1 mice with initiation by DMBA and promotion by twice weekly applications of TPA, retinyl palmitate provided in the diet caused a dose-dependent inhibition of the number and weight of papillomas at 21 weeks, reaching 65% in the group given 350 000 IU per kg of diet (Gensler *et al.*, 1987). In male rats given 0.05% BBN as carcinogen followed by 5% sodium saccharin as promoter, a diet containing 0.05% retinyl acetate suppressed saccharin-induced colony growth of bladder cells in soft agar, suggesting that vitamin A has anti-tumour-promoting effects on rat bladder carcinogenesis (Kanamaru *et al.*, 1988).

In a two-stage rat hepatocarcinogenesis model with DEN as the initiator and either 3,3',4,4'-tetrachlorobiphenyl or 2,2',4,4',5,5'-hexachlorobiphenyl as promoter, a diet containing 100 000 IU of retinyl palmitate decreased the number and volume of hepatic foci compared with animals on a diet with little or no retinyl palmitate. Thus, retinyl palmitate protected against preneoplastic changes enhanced by the tumour promoters (Berberian *et al.*, 1995).

One biomarker of genotoxic damage due to carcinogens is the formation of micronuclei in epithelial cells. A reduced frequency of micro-nucleated mucosal cells and remission of leukoplakias was seen following twice weekly administration of vitamin A (100 000 or 200 000 IU per week) for 3–6 months to chewers

of tobacco-containing betel quids. The development of new leukoplakias was also inhibited. After vitamin A administration was terminated, micro-nucleated cells and leukoplakia recurred in the oral cavity of chewers who continued to use tobacco throughout the trial period. Vitamin A given at a level of 50 000 IU per week maintained the frequency of micronucleated mucosal cells at low levels for at least 12 months after the trial (Stich *et al.*, 1991a,b).

The mechanism of suppression of tumour promotion is not clear, but may involve inhibition of the transformation of normal cells, or inhibition of proliferation of initiated or premalignant cells such that the premalignant lesion is not converted to a malignant one or the promotion step is prolonged.

Decreased levels of retinol or retinyl esters have been detected in premalignant lesions or tumours. For example, the vitamin A concentration in UV-induced tumours in mouse skin was significantly lower than in perilesional epidermis in mice consuming diets with or without vitamin A supplementation (20 000 IU/kg). The largest difference between the levels in tumour and epidermis was observed in the vitamin A-supplemented group. The low vitamin A content of the tumours was due to a marked reduction in the retinyl ester fraction, whereas the retinol content of the tumours actually increased two-fold. It was suggested that interconversion of retinol to retinyl esters may be disturbed in murine photo-carcinogenesis (Berne *et al.*, 1989). Serum levels of vitamin A were significantly decreased in 50 cases of oral leukoplakia compared with 50 normal controls (Ramaswamy *et al.*, 1996), but intralesional vitamin A levels were not measured in this study. In 34 patients with HPV infection of the cervix and in 40 patients with CIN (cervical intra-epithelial neoplasia) III, retinyl palmitate concentration was extremely low in CIN III tissue compared with normal cervical epithelium and HPV-infected tissue. In this study there was no significant difference in serum vitamin A levels between the two groups. It was concluded that the reduction of retinyl palmitate in CIN III is a local process and that a local supplementation of vitamin A might contribute to prevention of cervical neoplasia (Volz *et al.*, 1995).

4.3.1.2 Inhibition of transformation

As described in Section 4.2.3.1, retinol inhibits the transformation of cultured cells exposed to carcinogens and tumour promoters. Treatment of postconfluent cultures of mouse C3H 10T1/2 fibroblasts with certain carcinogens causes transformation to focus-forming cells, and retinyl acetate inhibits this transformation (Mordan, 1989). Areca nut (used in betel quids) has been linked to the high incidence of precancerous oral lesions and oral cancers in India. The formation of bovine papillomavirus (BPV) DNA-induced transformed foci was enhanced approximately tenfold in cultured mouse C3H 10T1/2 cells transfected with the plasmid pdPBV-1 harbouring BPV DNA and exposed to areca nut extracts. This transformation was completely suppressed when 1 µmol/L retinol was added to the areca nut extract. This was proposed as a mechanism of chemoprevention by vitamin A in betel quid chewers (Stich & Tsang, 1989).

4.3.1.3 Inhibition of DNA synthesis and cell proliferation

The transformation of density-arrested, initiated C3H 10T1/2 cells by certain carcinogens was correlated with the mitogenic response of the preneoplastic cells to platelet-derived or epidermal growth factor. Stimulation by serum of DNA synthesis in carcinogen-treated C3H 10T1/2 cells was inhibited by retinyl acetate, presumably by blockage of the G_0 to G_1 transition in the mitotic response of initiated cells to platelet growth factors (Mordan, 1989).

Retinol inhibited DNA synthesis and growth of MCA-induced squamous cell carcinomas in Swiss male albino mice and basal cell carcinomas in inbred Sprague–Dawley rats (Lupulescu, 1986). In another study, female Sprague–Dawley rats were treated with either solvent, DMBA or MNU at 50 days of age and were placed on a retinyl acetate-containing diet at 57 days of age. [^{3}H]Thymidine incorporation into purified DNA from mammary parenchymal cells was determined. Retinyl acetate effectively inhibited mammary cell DNA synthesis in both MNU- and DMBA-treated animals compared with those on control diet (Mehta & Moon, 1980).

4.3.1.4 Inhibition of ornithine decarboxylase induction

Cell proliferation and transformation require accelerated polyamine biosynthesis and elevated levels of ornithine decarboxylase (ODC), the rate-limiting enzyme in this reaction chain. Tumour promoters enhance ODC activity and retinoids suppress this induction. In an animal model for oral carcinogenesis, DMBA stimulated ODC activity and vitamin A inhibited late-phase ODC induction *in vivo* and prevented carcinogenesis (Calhoun *et al.*, 1989). That retinol also inhibited ODC induction in mouse skin was indicated by the finding that citral (3,7-dimethyl-2,6-octadienal) inhibits the oxidation of retinol to retinoic acid in mouse epidermis on local application. Citral treatment decreased the ability of retinol to inhibit the induction of epidermal ODC activity by TPA (Connor, 1988). In rats, a single intraperitoneal injection of TPA resulted in a transient increase in liver ODC activity. This increase was inhibited when retinyl acetate was injected one hour before TPA (Bisschop *et al.*, 1981). The inhibition by retinoids of the tumour promoter-induced increase in ODC activity was correlated with suppression of carcinogenesis (Verma *et al.*, 1979).

4.3.1.5 Restoration of normal differentiation

Carcinogenesis is characterized by aberrant differentiation, which is manifested by either blockage of cells at an early stage of differentiation or by redirection of differentiation towards an abnormal pathway. Retinol and retinyl esters have been reported to maintain proper differentiation in many epithelial tissues and to reverse abnormal differentiation in premalignant and malignant cells. Vitamin A deficiency is associated with loss of mucociliary differentiation and metaplastic changes to keratinizing squamous epithelium *in vivo* and in long-term rat tracheal organ cultures (Clark *et al.*, 1980). In the presence of retinyl acetate, normal mucociliary epithelium can be maintained for several months in organ culture of tracheal explants. Squamous metaplastic epithelium, which develops in vitamin A-deficient organ cultures, reverts to mucus-secreting tissue (Clark & Marchok, 1979; Clark *et al.*, 1980). The reversal of metaplasia *in vivo* was associated with prevention of lung carcinogenesis (Saffiotti *et al.*, 1967).

4.3.1.6 Restoration of gap junctional communication

An early event in cellular transformation is loss of intercellular communication via gap junctions. Retinol and retinyl esters enhance gap-junctional communication in carcinogen-initiated C3H 10T1/2 cells and suppress neoplastic transformation of these cells. A good correlation between these two events was shown by their overlapping responses to different retinoid doses. These findings led to the proposal that enhanced junctional communication may be an important mechanism for the chemopreventive action of retinol (Hossain *et al.*, 1989). In tracheal epithelial cells cultured in the absence of vitamin A, cigarette-smoke condensate inhibited intercellular communication. However, when retinol was added to tracheal epithelial cells before or simultaneously with exposure to cigarette-smoke condensate, it counteracted the inhibitory effect of the condensate on intercellular communication (Rutten *et al.*, 1988b).

4.3.1.7 Inhibition of prostaglandin production

Excessive production of prostaglandins has been correlated with tumour promotion. Expression of the enzyme cyclooxygenase-2 (Cox-2), which catalyses the synthesis of prostaglandins, can be induced by growth factors and tumour promoters and is up-regulated in transformed cells and tumours. Therefore, Cox-2 inhibition has potential relevance as a mechanism of chemoprevention. A few studies have demonstrated that retinol can suppress prostaglandin biosynthesis and this effect might mediate suppression of the growth of premalignant and malignant cells, thereby inhibiting carcinogenesis. The production of prostaglandin in normal tissues has been found to be regulated by retinol. Production of prostaglandin E_2 from arachidonic acid in bovine seminal vesicles and kidney was considerably inhibited by retinol, whereas the production of hydroxyeicosatetraenoic acid was inhibited to a smaller extent. Thus, it appears that retinol

influences both the cyclooxygenase and lipoxygenase pathways (Halevy & Sklan, 1987). Similarly, both retinol and retinoic acid inhibited the oxidation of arachidonic acid to prostaglandin E_2 in squamous carcinoma cells of the tongue (SCC-25) (ElAttar & Lin, 1991). Treatment of oral epithelial cells with TPA enhanced transcription of Cox-2 and increased production of prostaglandin E_2. These effects were inhibited by retinyl acetate and retinoic acid (Mestre *et al.*, 1997).

4.3.1.8 Modulation of cell adhesion and migration

Cell migration and invasion through the basement membrane are important for the conversion of advanced dysplastic lesions to malignant neoplasms. Inhibition of invasion may therefore suppress this conversion. In this context, it is noteworthy that a two-day pretreatment with retinyl acetate (0.09 and 3 µg/mL) inhibited the migration and invasion of A549 human lung carcinoma cells *in vitro* through human amnion basement membrane and the degradation of basement membrane components. This effect was accompanied by a significant decrease in type IV collagenase activity. It has been reported that retinoic acid can inhibit the expression of various basement membrane degradative enzymes (e.g., collagenase, stromelysin) at the level of transcription (Nakajima *et al.*, 1989; Nicholson *et al.*, 1990). These findings suggest that retinol may suppress the expression of invasive phenotype in carcinoma *in situ* and thereby prevent the development of a malignant lesion from a premalignant one (Fazely *et al.*, 1988).

4.3.2 Inhibition of angiogenesis

The progressive growth of solid tumours depends on the development of new blood vessels, a process known as neovascularization or angiogenesis. Tumour cells secrete factors that induce the directed migration and proliferation of endothelial cells from capillaries in the normal tissue, which eventually differentiate and form vessels around and within tumours. In rabbits, intramuscularly injected vitamin A inhibited angiogenesis induced in the eyes by intracorneal implants of carcinoma, and also resulted in inhibition of tumour growth at the implantation site (Arensman & Stolar, 1979). More recent studies with retinyl acetate and retinoic acid have demonstrated anti-angiogenic effects of retinoids in an assay using 4.5-day chorioallantoic membranes of chick embryo (Oikawa *et al.*, 1989). Retinoic acid causes large- and small-vessel endothelial cells to become refractory to stimulation of migration by either tumour-conditioned media or purified angiogenic factors (α-fibroblast growth factor (αFGF), bFGF, vascular endothelial growth factor, PDGF, TGFβ-1 and interleukin-8) without affecting cell proliferation. Retinoic acid (1 mg/kg/day) inhibited a neovascular response to tumour in the cornea of rats (Lingen *et al.*, 1996). These results indicated that the acid can affect directly both tumour cells and endothelial cells and thereby suppress the formation of new blood vessels *in vivo*. Although the latter study was not performed with retinol or retinyl acetate, the data overall suggest that vitamin A may exert its inhibitory effect on carcinogenesis and tumour growth *in vivo* by modifying the normal vascular response to neoplastic tissue.

4.3.3 Modulation of immune responses

Epidemiological studies suggest that vitamin A plays an important role in immune responses in adults and children. Vitamin A deficiency leads to impaired antibody responses to protein antigens, changes in lymphocyte subpopulations and altered T- and B-cell proliferation and function (Semba, 1994; Tomita, 1983). The immune defect caused by vitamin A deficiency may be due to alterations in lymphocyte cell membrane glycoproteins, an adverse effect on helper T-cell function, or some other mechanisms (Rumore, 1993). Vitamin A and its metabolites are immune enhancers that potentiate antibody responses to T-cell-dependent antigens and increase lymphocyte proliferation responses to antigens and mitogens (Semba, 1994). Vitamin A may boost immune responses in the elderly, in persons with high exposure to ultraviolet light, in patients who have undergone surgery, and in persons with parasitic infection (Rumore, 1993). A mechanism to help explain the anticancer action of vitamin A might be immunosuppression during deficiency or immuno-enhancement with high dietary intake. *In vitro*, retinol suppressed

T-lymphocyte functions, whereas high dietary levels of vitamin A enhanced macrophage functions. Enhancement of immune functions by high intake of vitamin A provides a mechanism to explain in part the decreased carcinogenesis (Watson, 1986).

Antitumour effects of vitamin A in mice have been related to immune enhancement in a number of systems. In CBA mice, a diet enriched in retinyl acetate (up to 0.8 g/kg diet) increased the ability of the immune system to respond effectively to tumour antigens and to suppress tumour growth in immunized mice. These effects could be explained partially by an increase in T-cell-mediated cytotoxicity but not by changes in natural killer (NK) cell activity (Malkovsky *et al.*, 1983). Antitumour activity could be increased even without immunization in mice placed on a retinyl acetate-enriched diet for at least three weeks (Malkovsky *et al.*, 1984). Diets with high levels of retinyl palmitate (4000–650 000 IU/kg diet) administered to CD-1 mice for 7 to 10 weeks increased mitogenic response in splenocytes, and enhanced phagocytic ability and tumoricidal activity of peritoneal macrophages, but did not enhance NK activity (Moriguchi *et al.*, 1985).

The administration of retinyl palmitate in the drinking water of BALB/c mice at 200, 500 or 1000 IU per mouse per day increased proliferative response to concanavalin A, *Escherichia coli* lipopolysaccharide and interleukin-2, and the production of lymphokines after 60–90 days. The growth of three distinct transplantable tumours was impaired when the tumour cells were transplanted on day 75 (Forni *et al.*, 1986). Similar results were reported in another syngenic murine tumour system *in vivo* in which intraperitoneal administration of vitamin A suppressed the growth of tumour cells inoculated subcutaneously (Tomita, 1983). [The Working Group was unclear about the nature and stability of the retinyl palmitate preparation that was apparently 'dissolved' into the drinking water.]

More relevant to chemoprevention than the above studies is the report that dietary retinyl palmitate administered at 350 IU/g of diet for 22–30 weeks to CD-1 mice, which had been given an initiating treatment with DMBA followed by TPA promotion, enhanced immune response and prevented skin tumour development. Mice on this diet exhibited an increase in the number of peritoneal macrophages and in their cytotoxic capacity. This enhanced immune response may have contributed to skin tumour prevention (Watson *et al.*, 1987).

Immunomodulatory effects of various retinoids have been demonstrated, both *in vivo* and *in vitro*, in murine and human thymocytes, human lung fibroblasts, Langerhans' cells, tumour cells and NK cells, and peripheral blood mononuclear cells. For example, retinol or retinoic acid (0.1 µmol/L; 48 h) acted as co-stimulators for human peripheral blood mononuclear cells in serum-free medium. Retinol increased the proliferation of such cells from 32 healthy individuals, which had been stimulated with anti-CD3 antibodies 48 h earlier. The increased proliferative response was mediated via the clonotypic T-cell receptor-CD3 complex and correlated with the up-regulation of T-lymphocyte surface adhesion/activation markers (CD18, CD45RO and CD25) and cytokines (interleukin-2 and interferon-gamma) at both the mRNA and protein levels. The authors suggested that retinoids may be used as therapeutic agents in immune system deficiencies that do not affect the clonotypic T-cell receptor (Allende *et al.*, 1997). Lipopolysaccharide-induced release of tumour necrosis factor (TNF) by human peripheral blood monocytes was suppressed in vitamin A-free medium and addition of retinol (1 µmol/L) resulted in the release of TNF-like activity into the culture supernatant after three days of culture (Turpin *et al.*, 1990).

Immune stimulation in humans *in vivo* has been demonstrated in a few studies. Retinyl palmitate was given for up to seven treatment courses during a period of 60 weeks to nine male patients with metastatic unresectable squamous cell carcinoma of the lung. An immune potentiating effect of the vitamin A therapy was indicated by an increase of lymphocyte blastogenesis response to phytohaemagglutinin and increased delayed cutaneous hypersensitivity reactions compared with the pretreatment values (Micksche *et al.*, 1977). Additional aspects of immune enhancement were observed after

treatment of patients suffering from chronic lymphocytic leukaemia with vitamin A (100 000 IU daily for two weeks). This resulted in enhancement of antibody-dependent cell-mediated cytotoxicity, NK cell activity and blastogenic response to plant mitogens (Gergely *et al.*, 1988).

These findings suggest that vitamin A may contribute to the prevention of cancer by augmenting an immunological response against tumour cells in the early stages of carcinogenesis. However, it should be noted that a recent report indicated a suppressive effect of supplemental vitamin A (800 µg retinyl palmitate per day) on cell-mediated immune response in an older population (Fortes *et al.*, 1998).

4.3.4 Other mechanisms

Apoptosis may be induced by vitamin A, although current information is limited (Maziere *et al.*, 1997). Vitamin A might also affect any of the processes discussed above by serving as an antioxidant, although many more effective antioxidants, such as vitamin E and ascorbic acid, exist in biological systems.