Immunology 1979 36 449

Contact sensitivity and the DNA response in mice to high and low doses of oxazolone: low dose unresponsiveness following painting and feeding and its prevention by pretreatment with cyclophosphamide

G. L. ASHERSON, M. A. C. C. PERERA & W. R. THOMAS *Division of Immunological Medicine, Clinical Research Centre, Northwick Park, Harrow, HA1 3UJ*

Received 22 *May* 1978; *accepted for publication* 28 *June* 1978

Summary. Cyclophosphamide was used to assess the role of suppressor cells in the contact sensitivity reaction. A single painting with 300 μg and 30 μg oxazolone produced poor contact sensitivity reactions (ear swelling). Cyclophosphamide (200 mg/kg) 2 days before painting increased the response to the lower doses but had less effect on the response to 3 mg oxazolone. A single feed with 10 mg oxazolone caused strong contact sensitivity while lower doses (10–1000 μg) caused poor responses. Cyclophosphamide increased the response to the lower doses but not to the highest dose of oxazolone. These results suggested that the poor response to painting and feeding lower doses of oxazolone was due to a suppressor system which was sensitive to cyclophosphamide. A different result was obtained when contact sensitivity was measured by arrival of radioactively labelled cells. Cyclophosphamide had the greatest effect on cell arrival when high doses were fed. This indicates that ear swelling and cell arrival measure separate aspects of the contact sensitivity response. The lower doses of oxazolone, which caused little contact sensitivity, reduced the response to a standard immunizing dose. This low dose unresponsiveness occurred after either painting or feeding (Chase–Sulzberger phenomenon). It did not occur in mice treated with cyclophosphamide before the first exposure to oxazolone. This suggested that the low dose unresponsiveness was due to suppressor cells. The response to oxazolone was also assessed by DNA synthesis in the regional lymph nodes. A small dose of oxazolone (30 μg) caused a peak of DNA synthesis on day four while a high dose (3 mg) caused a peak on day three. Pretreatment with cyclophosphamide depressed the response to 30 μg although it increased contact sensitivity. The secondary response was smaller than the primary on days 3, 4 and 5 after immunization but larger on day two. The depression but not the increase was prevented by cyclophosphamide and was probably due to a suppressor system.

Correspondence: Dr G. L. Asherson, Division of Immunological Medicine, Clinical Research Centre, Northwick Park, Harrow HA1 3UJ, Middlesex.

0019-2805/79/0300-0449$02.00

INTRODUCTION

When mice are painted on the skin with high doses of contact sensitizers they develop strong contact sensitivity and IgM, IgG and IgE antibody (Thomas, Asherson & Watkins, 1976; Takahashi, Nishikawa, Katsura & Izumi, 1977). Contact sensitivity also occurs in mice fed high doses of the contact sensitizer, oxazolone (Asherson, Zembala, Perera, Mayhew & Thomas, 1977). These procedures induce two types of suppressor cells both of which are sensitive to cyclophosphamide. There are B cells with surface immunoglobulin which block the effector stage of

contact sensitivity (Zembala, Asherson, Noworolski & Mayhew, 1976; Asherson *et al.*, 1977). There are also T cells which depress DNA synthesis, the appearance of T-cell cytotoxicity and the production of IgG antibody (Wood, Asherson, Mayhew, Thomas & Zembala 1977; Thomas, Watkins & Asherson, 1978). Experiments on delayed hypersensitivity to sheep red cells suggest that there may be an additional type of cyclophosphamide-sensitive T suppressor cell which blocks the effector stage of delayed hypersensitivity (Mitsouka, Baba & Morikawa, 1976; Liew, 1977). All these T cells are different from the T suppressor cells induced by the injection of picrylsulphonic acid which are sensitive to adult thymectomy (Asherson, Zembala, Mayhew & Goldstein, 1976).

This paper investigates two factors which may affect the induction of suppressor cells, the dose of contact sensitizer and the route of immunization. It shows that doses of contact sensitizer, which cause poor primary responses when painted on the skin, depress the contact sensitivity response to a standard second dose. This depression is a form of low dose unresponsiveness. It does not occur after pretreatment with cyclophosphamide which suggests that the unresponsiveness is not due to classical (deletion) tolerance but to suppressor cells. This paper also shows that the poor primary response to intermediate doses of antigen, is greatly increased by pretreatment with cyclophosphamide. This suggests that suppressor mechanisms are involved in controlling the size of the primary response.

This paper studies the response to feeding contact sensitizer and shows that large doses cause immunity while lower doses cause low zone unresponsiveness (Chase–Sulzberger phenomenon). This does not occur after pretreatment with cyclophosphamide.

Finally this paper investigates the effect of the dose of contact sensitizer in the primary response on the DNA synthesis in the regional lymph nodes during the secondary response. It shows that priming with contact sensitizer reduces DNA synthesis in the secondary response. This depression does not occur after pretreatment with cyclophosphamide and is probably due to suppressor cells.

MATERIALS AND METHODS

Animals

CBA mice were bred locally or purchased. Mice of the same sex were used in any one experiment. The general methods are given in Asherson *et al.* (1977).

Contact sensitivity

Five mice were used in each group. A volume of 0·1 ml of various concentrations of oxazolone (4-ethoxymethylene-2-phenyloxazolone, British Drug Houses) in ethanol was applied to the skin of the abdomen and lower chest from which hair had been removed by electric clippers. One drop was also applied to each of the forepaws. The oxazolone was dissolved shortly before use in ethanol and dilutions made in glass test-tubes. Six days later the ears were challenged with 1% oxazolone in olive oil. The increment in ear thickness was measured at 24 h with an engineers' micrometer in units of 10^{-5} m. One day later the mice were painted again with a standard immunizing dose of 3 mg oxazolone on the skin and forepaws and the ears challenged after a further 7 days with 0·5% oxazolone and the increment of ear thickness again measured at 24 h. In this protocol the ears were challenged on day 6 and again on day 14. Preliminary experiment showed that similar results were obtained if the first ear challenge was omitted.

Feeding oxazolone

Mice were fed under ether anaesthetic with 10 mg or smaller amounts of oxazolone dissolved in 0·5 ml olive oil using a fine plastic tube. The mice were tested for contact sensitivity 6 days after the last feed by challenging the ears. This procedure gives rise to strong contact sensitivity in CBA mice and slight contact sensitivity in TO mice with high doses of oxazolone (10 mg).

Index of cell arrival

Mice were injected with fluorodeoxyuridine followed by [^{125}I]-iododeoxyuridine 2–3 h before challenge of the right ear of groups of five mice with oxazolone. After 24 h both ears were cut off at the junction where the cartilage becomes thicker, and given three daily washes in 70% alcohol. Their radioactivity was measured. The index of cell arrival is the ratio of the radioactivity in the painted ear to that in the unpainted ear (Vadas, Miller, Gamble & Whitelaw, 1975).

Cyclophosphamide

Cyclophosphamide (Endoxana, WB Pharmaceuticals, Berks.) at a dose of 200 mg/kg was injected intra-

peritoneally 2 days before the first exposure to oxazolone. In the text the increase in contact sensitivity caused by cyclophosphamide was calculated after subtracting the mean value of the negative control group.

DNA synthesis in the regional lymph nodes

Groups of three to four mice were painted with oxazolone. Painting of the forepaws was needed to obtain good responses in the shoulder girdle nodes. At various times afterwards they were injected intraperitoneally with 0·2 ml 10^{-7} M fluorodeoxyuridine (Fluka) and 30 min later with 2 μCi [^{125}I]-iododeoxyuridine (Amersham Radiochemicals). The regional shoulder girdle and inguinal lymph nodes were harvested 2 h later, given three daily washes in alcohol and their radioactivity counted. The ^{125}I incorporation was expressed as a percentage of the injected radioactivity and used as a measure of DNA synthesis. The cells were taken at 2 h because the uptake of radioactivity is complete at that time and the short pulse minimized cell movement after the labelling of the cells.

Statistics

Standard deviation (SD) was used as a measure of scatter. The length of the vertical line in one direction on the graphs indicates 1 SD. Significance was assessed by Student's *t*-test.

RESULTS

Contact sensitivity in mice painted with oxazolone

Primary contact sensitivity after a single painting with oxazolone and the effect of cyclophosphamide. The first experiments investigated contact sensitivity following immunization with serial ($\log_{10}$) dilutions of oxazolone applied to the skin and the effect of cyclophosphamide on the response. Six days after immunization the ears were challenged and contact sensitivity was assessed by the ear swelling 24 h later. Table 1 (Exp. 1) shows that 3 mg and 300 μg oxazolone caused strong contact sensitivity, 30 and 3 μg had an intermediate effect while 300 ng was inactive. Pretreatment with cyclophosphamide (200 mg/kg) before immunization had little effect on the response to the two highest doses of oxazolone but caused an increase of 2·7 fold in the response to 30 μg. The same pattern was seen in Exp. 2. It was concluded that cyclophosphamide increased the response to intermediate doses of oxazolone much more than the response to high doses.

Low dose unresponsiveness to oxazolone and the effect of cyclophosphamide. As part of the previous experiment mice primed with serial dilutions of oxazolone were re-immunized 7 days later with the

Table 1. The primary and secondary contact sensitivity response in mice painted with various doses of oxazolone and the effect of pretreatment with cyclophosphamide.

	Primary response			Secondary response		
		Contact sensitivity (ear swelling)			Contact sensitivity (ear swelling)	
Exp. No.	Dose used for primary (μg)	Without cyclophosphamide	With cyclophosphamide	Dose used for secondary (mg)	Without cyclophosphamide	With cyclophosphamide
1	3000	10·7± 1·53	10·2± 0·94	3	7·4± 1·13	8·5± 2·45
	300	7·5± 1·40	9·1± 1·66	3	5·5± 1·28	7·5± 2·44
	30	4·7± 1·36	9·1± 0·53	3	1·7± 0·34	8·9± 1·33
	3	3·0± 0·40		3	2·9± 0·94	
	0·3	1·9± 0·33		3	5·2± 1·74	
	—	2·1± 0·31		3	6·9± 1·18	
2	30	9·3± 1·83	17·5± 0·69	3	7·5± 0·47	11·0± 0·92
	3	4·5± 1·37	10·3± 1·54	3	2·9± 0·51	12·2± 0·10
	0·3	3·7± 1·22	3·3± 0·12	3	10·6± 0·77	12·3± 0·75
	—	1·9± 1·06				

Groups of mice were treated with cyclophosphamide (200 mg/kg) or left untreated. Two days later they were immunized by painting the skin with various doses of oxazolone. Contact sensitivity was assessed 6 days later by ear swelling (units of 10^{-5} m) ± standard deviation. The mice were then painted with a standard large dose of oxazolone and contact sensitivity reassessed after a further 7 days.

standard large dose (3 mg). Contact sensitivity was assessed seven days later. Table 1 (Exp. 1) shows that priming with 30 μg and 3 μg depressed the secondary response while 300 ng had no effect. Pretreatment with cyclophosphamide prevented this inhibition. The same pattern was seen in Exp. 2. It was concluded that oxazolone caused low dose unresponsiveness in normal mice but not in mice pretreated with cyclophosphamide.

Contact sensitivity in mice fed oxazolone by gastric tube

Primary contact sensitivity after a single feed with oxazolone and the effect of cyclophosphamide. Contact sensitivity can be produced in CBA mice by feeding a large dose of oxazolone. Figure 1 shows the contact sensitivity response 6 days after a single feed with serial ($\log_{10}$) dilutions of oxazolone and the effect of cyclophosphamide given 2 days before the feed. There was a linear log dose response curve between 0·1 and 10 mg. Pretreatment with cyclophosphamide before immunization had little effect on the response to the highest dose of oxazolone but caused a 6·6 fold increase in the response to 10 μg.

Contact sensitivity was also assessed by the index of cell arrival. The index was significantly raised (1·7) in mice fed 10 mg oxazolone and there was a smaller rise in mice fed lower doses when cyclophosphamide was not used (Table 2). The index in unimmunized mice was 1·2. In contrast, mice given cyclophosphamide and then fed, showed large

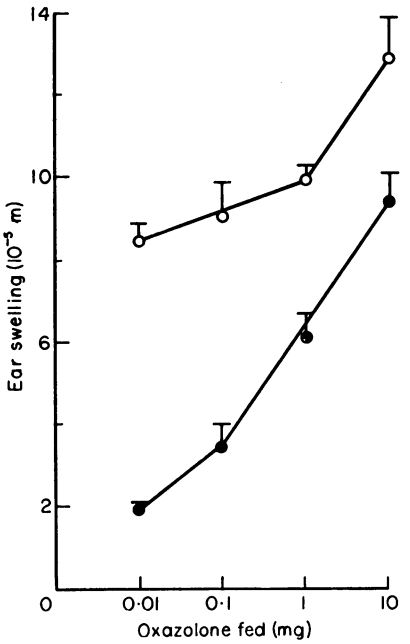

Figure 1. The primary contact sensitivity response in mice fed various doses of oxazolone and the effect of pretreatment with cyclophosphamide. The horizontal axis shows the amount of oxazolone fed on a logarithmic scale. The vertical axis shows contact sensitivity in the primary response measured by increase of ear thickness in units of 10^{-5} m on challenge six days later. The upper curve (open symbols) shows the response in mice given cyclophosphamide 2 days before the first feed. The lower curve (filled symbols) is for no cyclophosphamide. The negative controls with and without cyclophosphamide were 1·1 and 1·8. The index of cell arrival for the same experiment is shown in Table 2.

Table 2. The primary and secondary contact sensitivity response, assessed by cell arrival in mice fed various doses of oxazolone and then refed with 10 mg oxazolone and the effect of pretreatment with cyclophosphamide. The data for the contact sensitivity in these mice, assessed by ear swelling are shown in Figs 1 and 2. The data for the primary and secondary response are based on separate experiments. The index of arrival 6 days after a *single* feed of 10 mg oxazolone in the 'secondary' experiment was 2·5±0·53.

Primary response			Secondary response		
Dose fed for primary (μg)	Index of cell arrival		Dose fed for secondary (mg)	Index of cell arrival	
	Without cyclophosphamide	With cyclophosphamide		Without cyclophosphamide	With cyclophosphamide
10,000	1·7±0·27	7·3±1·03	10	2·6±0·51	4·2±1·23
1000	1·3±0·20	4·3±1·47	10	1·6±0·49	2·3±0·37
100	1·3±0·20	3·4±1·10	10	1·5±0·41	3·0±0·45
10	1·4±0·31	2·6±0·47	10	2·1±0·49	2·7±0·38
—	1·2±0·30	1·3±0·42	—	1·4±0·28	1·6±0·24

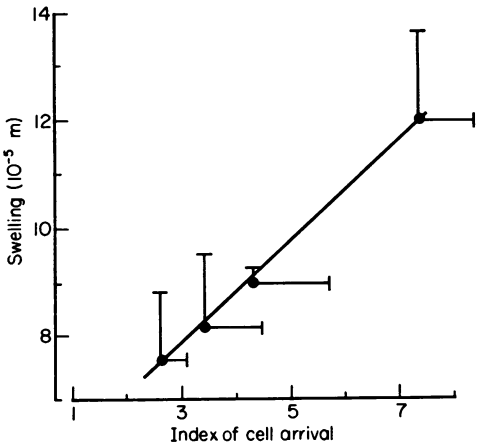

Figure 2. Relationship between index of cell arrival and ear swelling in the primary response in mice pretreated with cyclophosphamide. Mice were given 200 mg/kg cyclophosphamide. Two days later they were fed various doses of oxazolone and contact sensitivity assessed by ear swelling and the index of cell arrival 6 days later. This graph shows the linear relationship between the index of cell arrival (horizontal axis) and the increment of ear thickness (vertical axis). The amount of antigen fed to obtain these points was (reading from left to right) 0·01, 0·1, 1 and 10 mg. These data are taken from Fig. 1 and Table 2.

indices ranging from 7·3 in mice given 10 mg to 2·6 in mice given 10 μg. Figure 2 shows that there was a linear relationship between the ear swelling and the index of cell arrival in these mice. There was also a linear relationship between these parameters and the dose of oxazolone fed in the range 10 μg to 1 mg.

Low dose unresponsiveness after a single feed with oxazolone and the effect of cyclophosphamide. In the following experiment contact sensitivity was produced by feeding oxazolone. Mice which had been primed by feeding were immunized by feeding a standard large (10 mg) dose of oxazolone 10 days later. Contact sensitivity was assessed 6 days afterwards. Figure 3 shows that priming with 10 mg oxazolone increased the secondary response, 1 mg had little effect while lower doses caused a depression of about 80%. Pretreatment with cyclophosphamide prevented this low dose unresponsiveness. Table 2 shows that low dose unresponsiveness and its prevention by cyclophosphamide could also be demonstrated by the index of cell arrival.

In another experiment low dose unresponsiveness was demonstrated in mice primed by feeding and

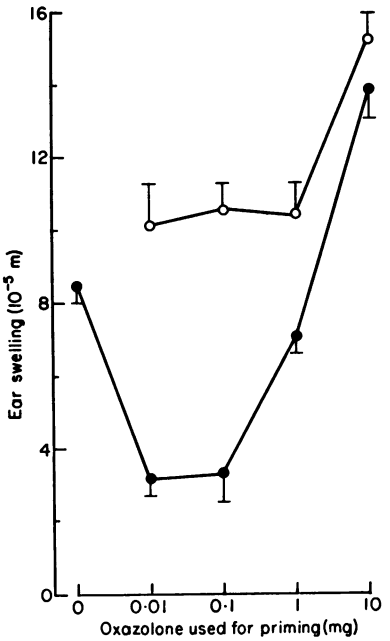

Figure 3. The secondary contact sensitivity response in mice fed various doses of oxazolone and then immunized by feeding with 10 mg oxazolone and the effect of pretreatment with cyclophosphamide. The horizontal axis shows the amount of oxazolone which was fed as the priming dose. The vertical axis shows contact sensitivity measured by ear swelling 7 days after a second feed of 10 mg oxazolone. The upper curve (open symbols) shows the response in mice given cyclophosphamide 2 days before the first feed. The lower curve (filled symbols) is for no cyclophosphamide. The swelling in mice which received no oxazolone was $1·4 \pm 0·48$, and $1·6 \pm 0·24$ in mice given cyclophosphamide. The index of cell arrival in the same experiment is shown in Table 2.

then immunized by painting the skin. Table 3 shows that this low dose unresponsiveness was specific and priming with 100 μg oxazolone had no effect on the response to picryl chloride.

DNA response in mice painted with oxazolone

Primary DNA response in the regional lymph nodes after a single painting with oxazolone. The observation that 3 μg oxazolone produced low dose unresponsiveness raised the question whether this dose of oxazolone caused DNA synthesis in the regional lymph nodes or other tissue. Figure 4 shows that painting with 3 μg caused a doubling of DNA synthesis in the regional lymph nodes which reached

Table 3. The effect of feeding oxazolone on the contact sensitivity response to painting with oxazolone and picryl chloride.

Feed (day 0)	Paint (day 10)	Challenge (day 16)	Contact sensitivity: Ear swelling	Contact sensitivity: Index of cell arrival
—	3 mg Ox	Ox (+ve control)	9·8±1·12	3·0±0·41
0·1 mg Ox	3 mg Ox	Ox	4·8±1·40	2·2±0·40
—	—	Ox (−ve control)	1·8±0·36	1·4±0·28
—	5 mg Pic	Pic (+ve control)	10·3±0·59	2·2±0·65
0·1 mg Ox	5 mg Pic	Pic	10·9±0·44	3·0±0·95
—	—	Pic (−ve control)	1·2±0·40	1·2±0·12

Ox refers to oxazolone and Pic to picryl chloride.

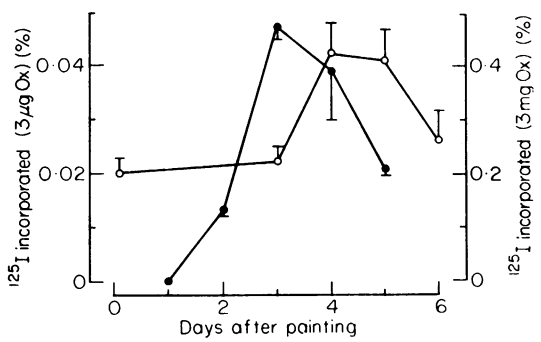

Figure 4. The primary DNA response in the regional lymph nodes following immunization by painting with high and low doses of oxazolone. The horizontal axis is the days after painting with oxazolone on which the lymph nodes were examined. The vertical axis shows the percentage incorporation of [^{125}I]-IdUR(iododeoxyuridine) which is a measure of DNA synthesis. The left hand axis refers to mice given 3 μg oxazolone (open symbols) and the right hand axis to mice given 3 mg oxazolone (filled symbols). The background DNA synthesis in mice which were not painted with oxazolone is shown on the extreme left (day 0).

a peak 4 and 5 days after immunization. There was no increase of DNA synthesis in the spleen, liver or lungs (data not shown). In contrast the response to 3 mg reached a peak on day 3. There was a brisk response in the spleen, liver and lungs. There was no response to 300 ng (data not shown).

Because the response to low doses reached a peak 4 days after painting, the response to serial ($\log_{10}$) dilutions of oxazolone was studied at that time. Figure 5 shows that there was an almost linear log dose response curve when 3–3000 μg oxazolone was used.

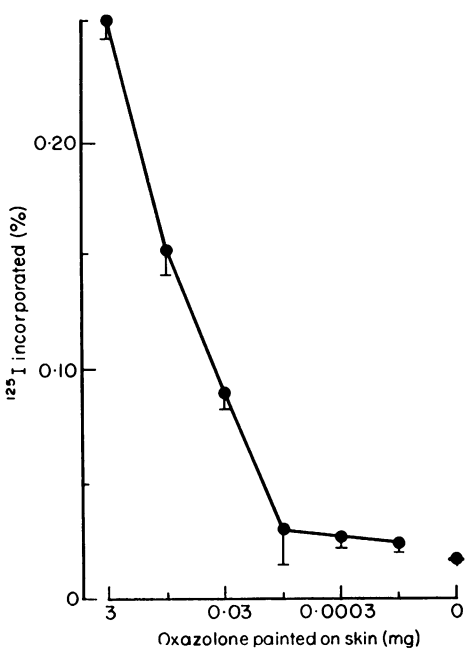

Figure 5. Relationship between the amount of oxazolone painted on the skin and DNA synthesis in the regional lymph nodes 4 days later. The horizontal axis shows the amount of oxazolone painted on the skin. The vertical axis shows the percent incorporation of [^{125}I]-IdUR which is a measure of DNA synthesis. The value for DNA synthesis in unpainted mice is shown on the extreme right.

Effect of cyclophosphamide on the primary DNA response. Painting with 30 μg oxazolone produced negligible contact sensitivity unless the mice were pretreated with cyclophosphamide (Table 1). This

Table 4. The effect of cyclophosphamide on the primary DNA response in the regional lymph nodes following painting with oxazolone.

Cyclophosphamide (day 2) (mg/kg)	Oxazolone (day 0) (μg)	DNA synthesis [^{125}I]-IdUR incorporation (%)	
		Day 3	Day 4
—	3000	0·40±0·012	0·29±0·084
200	3000	0·14±0·053	0·24±0·043
—	30	0·08±0·011	0·13±0·017
200	30	0·027±0·013	0·09±0·033
200	—	0·008±0·001	0·016±0·001
—	—	0·017±0·002	

raised the question of whether cyclophosphamide might increase the primary DNA response to low doses of sensitizer. Table 4 shows that pretreatment with cyclophosphamide reduced the DNA response to 30 μg oxazolone. It was concluded that the increase in contact sensitivity caused by cyclophosphamide was not associated with a general increase in cell proliferation in the regional lymph nodes.

Secondary DNA response to oxazolone in the regional lymph nodes. The finding that oxazolone may cause low dose unresponsiveness suggested that priming with oxazolone might depress the DNA response to a second painting 10 days later. Figure 6 shows that priming with 30 μg oxazolone reduced the response to painting 3, 4 and 5 days after the second painting but increased the response on day 2. Quite small doses of oxazolone depressed the DNA response to a second painting and priming with oxazolone in the dose range 3–3000 μg caused considerable depression of the secondary response on day 3 (Fig. 7).

Effect of cyclophosphamide on the secondary DNA response to oxazolone in the regional lymph nodes. A T suppressor cell which depresses DNA synthesis in transfer experiments, occurs in mice 3–4 days after immunization with picryl chloride. This cell is sensitive to pretreatment with cyclophosphamide. It is possible that the depression of the secondary DNA response caused by priming with oxazolone may be due to these suppressor T cells. One prediction of this view is that the depression of the secondary response should not occur in mice pretreated with cyclophosphamide. In the following experiment normal mice and mice pretreated with cyclophosphamide were used to investigate the effect of priming with 30 μg oxazolone on the secondary DNA response to 3 mg oxazolone. Table 5 shows an interesting contrast between the result obtained 2 and 3 days after the second painting. Priming increased the DNA response by 86% in normal mice on day two and there was a greater increase (153%) in mice pretreated with cyclophosphamide. In contrast priming reduced the response on day 3 by 49% in normal mice but had virtually no effect in

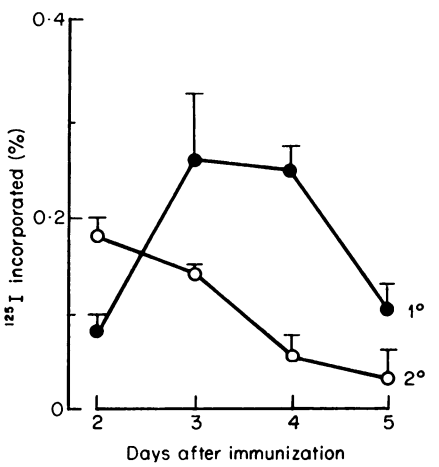

Figure 6. The effect of priming with oxazolone on the DNA response to a second painting with oxazolone. The upper line (filled symbols) shows the DNA response in the regional lymph nodes 2, 3, 4 and 5 days after painting with 3% oxazolone. The lower line (open symbols) shows the response to the same dose of oxazolone in mice painted 10 days previously with 30 μg oxazolone. The horizontal axis shows the day after painting with 3% oxazolone on which the regional lymph nodes were examined. The vertical axis shows the percent incorporation of [^{125}I]-IdUR.

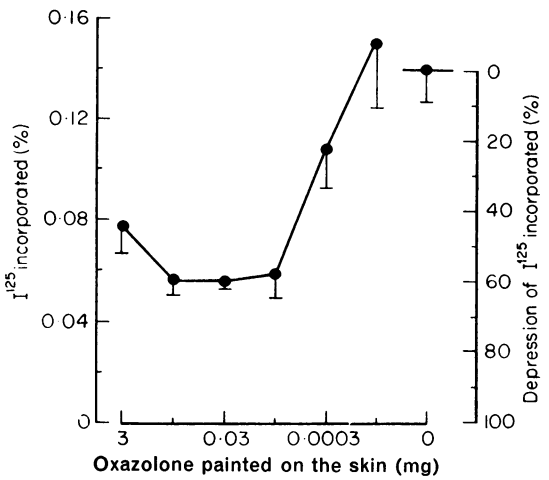

Figure 7. Relationship between the amount of oxazolone painted on the skin in the primary response and the depression of DNA synthesis in the regional lymph nodes on day 3 of the secondary response. The horiontal axis shows the amount of oxazolone painted on the skin in the primary response. The left hand vertical axis shows the percentage [^{125}I]-IdUR incorporation. The right hand axis shows the percentage *depression* of [^{125}I]-IdUR incorporation as compared with the primary response on day 3 (horizontal line on extreme right).

mice pretreated with cyclophosphamide. This supported the view that priming depressed DNA synthesis on day 3 of the secondary response through a cyclophosphamide sensitive system.

DISCUSSION

This paper describes the effect of the size and route of the first exposure to oxazolone on the primary and secondary immune response. This is part of a wider study of the conditions for the induction of effector and suppressor cells. Four conclusions are discussed. First, the inability of certain doses of oxazolone to produce strong contact sensitivity is not due to a failure to induce T effector cells but to the disproportionate induction of suppressor cells. Second, low dose unresponsiveness occurs after painting or feeding intermediate doses of oxazolone. Third, there are important similarities between the effects of painting and feeding oxazolone. In both cases a single high dose causes contact sensitivity while intermediate doses cause unresponsiveness. Finally, the DNA response in the regional lymph nodes on re-exposure to antigen is influenced by a suppressor system which is sensitive to cyclophosphamide.

Evidence that suppressor cells influence the primary contact sensitivity response

The response to a single painting or feeding depends on the dose of sensitizer. In both cases the highest dose causes strong contact sensitivity, while intermediate doses have much less effect. The unexpected finding is that pretreatment with cyclophosphamide causes the intermediate doses to produce almost as much contact sensitivity (ear swelling) as the highest dose.

Several workers have interpreted the increase of delayed hypersensitivity following treatment with cyclophosphamide as evidence for a suppressor system (Turk & Parker, 1973; Sy, Miller & Claman, 1977; Kerckhaert, Hofhuis & Willers, 1977). The effect of cyclophosphamide in the present system

Table 5. The effect of cyclophosphamide on the primary and secondary DNA response in the regional lymph nodes following painting with oxazolone.

Type of response	Cyclophosphamide (day 2) (mg/kg)	Oxazolone (day 0) (μg)	Oxazolone (day 10) (mg)	DNA synthesis [^{125}I]-IdUR incorporation (%) day 12 (+2)*	DNA synthesis [^{125}I]-IdUR incorporation (%) day 13 (+3)*
Primary	—	—	3	0·22±0·028	0·59±0·055
Secondary	—	30	3	0·41±0·044 (86%)†	0·30±0·030 (49%)‡
Primary	200	—	3	0·16±0·025	0·43±0·067
Secondary	200	30	3	0·43±0·067 (153%)†	0·39±0·079 (9%)‡

* Days after last immunization.
† Percentage increase of the secondary response in relation to the primary, i.e. (secondary−primary)/(primary).
‡ Percentage decrease of the secondary response in relation to the primary.

suggests that the limited contact sensitivity response to intermediate doses of oxazolone is not due to a primary inability of T cells to respond but to a suppressor system which blocks the development or expression of effector cells. In fact it is likely that a suppressor system sensitive to cyclophosphamide is responsible for the poor response to both supra-optimal and suboptimal doses of contact sensitizer (Sy *et al.*, 1977). It would be interesting to know if identical suppressor cells operate in these two situations.

This conclusion implies that the reason why high doses of contact sensitizer cause strong contact sensitivity is not self-evident and needs a detailed explanation. It is not due to the failure of high doses of contact sensitizer to induce T or B suppressors nor to a failure of T effector cells induced by high doses of antigen to respond to suppressor cells (Wood, Asherson, Mayhew, Thomas & Zembala, 1977; Zembala *et al.*, 1976). The most likely explanation is that the dose response curves for the production of suppressor and effector cells are slightly different and that high doses move the balance in favour of the effector cells. The finding that a strong immunogen like oxazolone induces both effector and suppressor cells is paralleled by the observation that strong immunogens induce suppressor cells to a greater extent than weak immunogens in a delayed hypersensitivity system (Scheper, Parker, Noble & Turk, 1977).

The concept that suppressor systems are important in determining the result of immunization is supported by the effect of pretreatment with cyclophosphamide in several immune systems. This agent increases the delayed hypersensitivity response to antigen in Freund's incomplete adjuvant, and enables proteins lightly conjugated with lipophilic groups to cause lasting delayed hypersensitivity without the use of Freund's adjuvant (Chiba, Otokawa & Egashira, 1976). It also enables highly conjugated dinitrophenyl-bovine gamma globulin to stimulate antibody production to bovine gamma globulin (Noble, Parker, Scheper & Turk, 1977). The implication is that the failure to develop a particular immune response is not due to a primary inability to respond to the antigen but to a suppressor system. A less likely alternative is that pretreatment with cyclophosphamide affects T cells directly and makes them more responsive to lower doses or different forms of antigen.

In some experiments contact sensitivity was assessed by an index which measures the arrival of labelled cells (Vadas *et al.*, 1975). In the primary response there is a linear relationship between ear swelling and the index of cell arrival in mice pretreated with cyclophosphamide. However there is much less agreement between the two measurements in mice fed oxazolone twice. We suspect that the contact sensitivity reaction is complex and that the index of cell arrival and ear swelling measure different aspects of the phenomenon. In fact the index depends on the cell arrival while swelling depends on oedema and induration and may be affected by fibrin deposition or alteration in ground substance (Colvin and Dvorak, 1975). Scheper, Noble, Parker and Turk (1977) also provide evidence that there are several components to the delayed hypersensitivity reaction.

Low dose unresponsiveness

The present findings show that low dose unresponsiveness occurs after either painting or feeding oxazolone. It also occurs in guinea-pigs painted with low doses of sensitizers (Lowney, 1965) or injected intradermally with immunizing doses providing the injection site is removed within 24 h (Macher & Chase, 1969). Low dose unresponsiveness occurs in tumour, transplantation and humoral systems (Bonmasser, Menconi, Goldin & Cudkowicz, 1974; Kilshaw, Brent & Thomas, 1974; Mitchison, 1964). The low dose unresponsiveness reported here is probably due to a suppressor system as it is prevented by cyclophosphamide.

Comparison between the effects of feeding and painting

Chase (1946) described unresponsiveness produced by feeding contact sensitizer to guinea-pigs (Chase-Sulzberger phenomenon). The contrast between unresponsiveness produced by feeding and the immunity produced by painting might suggest that these two procedures have very different effects. There are, however, important similarities. Both procedures induce T and B suppressor cells (Asherson *et al.*, 1977). They cause contact sensitivity when high doses are used and poor responses, which are greatly increased by cyclophosphamide, when lower doses are used. They also cause a low dose unresponsiveness which is prevented by cyclo-

phosphamide. In fact, the difference between painting and feeding, on the contact sensitivity response, is quantitative and not qualitative.

DNA response to antigen and its control by suppressor cells

The present results confirm an earlier observation that the secondary DNA response in the regional lymph nodes is smaller than the primary 3 days after immunization (Asherson, Zembala & Wood, 1974). This might suggest that the T cells which depress DNA synthesis in transfer experiments are responsible for the poor secondary response in the intact animal. Indirect evidence suggests that this is the case. The T cells which depress DNA synthesis are sensitive to cyclophosphamide. Likewise the depressed DNA response on day 3 of the secondary response does not occur in animals pretreated with cyclophosphamide. Moreover priming with antigen depressed the secondary response (on day 3) when elicited 10 or 20 days but not 30 days later (Asherson *et al.*, 1974 and unpublished data). Similarly T suppressor cells can be demonstrated in transfer experiments up to about day 16 but not later (Asherson, Wood and Mayhew, 1975).

It is interesting that day 2 of the secondary response is bigger than the primary response and that this phenomenon is not prevented by cyclophosphamide. This indicates that cyclophosphamide does not block all the effects of priming.

Low dose unresponsiveness may throw light on human reactions to drugs and infection. Its effect is that initial exposure to low doses of antigens may limit the severity of the response on subsequent exposure. The sensitivity of mice treated with cyclophosphamide to a few micrograms of oxazolone and a few thousand micro-organisms leads to speculation about the clinical features of congenital or acquired deficiency of the cyclophosphamide sensitive suppressor systems. Such individuals would be unduly prone to drug reactions and might have a higher incidence of staphylococcal boils as the severity of staphylococcal skin lesion in mice correlates with the intensity of the delayed hypersensitivity reaction to the organism (Easmon & Glynn, 1977). In fact the basic defect in some patients with allergic drug reactions may be a failure to develop suppressor cells and not a primary over-reactivity to antigen.

ACKNOWLEDGMENTS

M. A. C. C. Perera thanks the British Council and the Wellcome Trust for financial support.

REFERENCES

Asherson G.L., Wood P.J. & Mayhew B. (1975) Control of the immune response. I. Depression of DNA synthesis by immune lymph nodes cells. *Immunology*, **29**, 1057.

Asherson G.L., Zembala M., Mayhew B. & Goldstein A. (1976) Adult thymectomy prevention of the appearance of suppressor T cells which depress contact sensitivity to picryl chloride and reversal of adult thymectomy effect by thymus extract. *Europ. J. Immunol.* **6**, 699.

Asherson G.L., Zembala M., Perera M., Mayhew B. & Thomas W.R. (1977) Production of immunity and unresponsiveness in the mouse by feeding contact sensitizing agents and the role of suppressor cells in the Peyer's patches, mesenteric lymph nodes and other lymphoid tissues. *Cell. Immunol.* **33**, 145.

Asherson G.L., Zembala M. & Wood P.J. (1974) Control mechanisms of cell mediated immunity. The separate control of net DNA synthesis and of contact sensitivity skin reactions and the role of thymus-derived cells. *Monogr. Allergy*, **8**, 154.

Bonmasser E., Menconi E., Goldin A. & Cudkowicz G. (1974) Escape of small numbers of allogeneic lymphoma cells from immune surveillance. *J. nat. Canc. Inst.* **53**, 475.

Chase M.W. (1946) Inhibition of experimental drug allergy by prior feeding of sensitizing agent. *Proc. Soc. exp. biol. Med.* **61**, 257.

Chiba J., Otokawa M. & Egashira Y.A. (1976) Transient delayed type hypersensitivity induced by lightly lipid-conjugated BSA and its conversion by cyclophosphamide treatment in guinea pigs. *Japan J. Med. Sci. Biol.* **29**, 277.

Colvin R.B. & Dvorak H.F. (1975) Role of the clotting system in cell-mediated hypersensitivity. II. Kinetics of fibrinogen/fibrin accumulation and vascular permeability changes in tuberculin and cutaneous basophil hypersensitivity reactions. *J. Immunol.* **114**, 377.

Easmon C.S.F. & Glynn A.A. (1977) Effect of cyclophosphamide on delayed hypersensitivity to *Staphylococcus aureus* in mice. *Immunology*, **33**, 767.

Kerckhaert J.A.M., Hofhuis F.M.A. & Willers J.M.W. (1977) Influence of cyclophosphamide on delayed hypersensitivity and acquired cellular resistance to *Listeria monocytogenes* in the mouse. *Immunology*, **32**, 1027.

Kilshaw P.J., Brent L. & Thomas A.V. (1974) Specific unresponsiveness to skin allografts in mice. II. The mechanism of unresponsiveness induced by tissue extracts and antilymphocyte serum. *J. Immunol.* **17**, 57.

Liew F.Y. (1977) Regulation of delayed-type hypersensitivity. T suppressor cells for delayed-type hypersensitivity to sheep erythrocytes in mice. *Europ. J. Immunol.* **7**, 714.

Lowney E.D. (1965) Immunologic unresponsiveness appearing after topical application of contact sensitizers to the guinea pig. *J. Immunol.* **95**, 397.

MACHER E. & CHASE M.W. (1969) Studies of the sensitization of animals with simple chemical compounds. XII. The influence of excision of allergenic depots on onset of delayed hypersensitivity and tolerance. *J. exp. Med.* **129,** 103.

MITCHISON N.A. (1964) Induction of immunological paralysis in two zones of dosage. *Proc. Roy. Soc. B,* **161,** 275.

MITSOUKA A., BABA M. & MORIKAWA S. (1976) Enhancement of delayed hypersensitivity by depletion of suppressor T cells with cyclophosphamide. *Nature (Lond.)* **262,** 77.

NOBLE B., PARKER D., SCHEPER R.J. & TURK J.L. (1977) The relation between B cell stimulation and delayed hypersensitivity. The effect of cyclophosphamide pretreatment on antibody production. *Immunology,* **32,** 885.

SCHEPER R.J., NOBLE B., PARKER D. & TURK J.L. (1977) The value of an assessment of erythema and increase in thickness of the skin reaction for a full appreciation of the nature of delayed hypersensitivity in the guinea pig. *Int. Arch. Allerg.* **54,** 58.

SCHEPER R.J., PARKER D., NOBLE B. & TURK J.L. (1977) The relation of immune depression and B cell stimulation during the development of delayed hypersensitivity to soluble antigens. *Immunology,* **32,** 265.

SY M.S., MILLER S.D. & CLAMAN H. (1977) Immune suppression with supra-optimal doses of antigen in contact sensitivity I. Demonstration of suppressor cells and their sensitivity to cyclophosphamide. *J. Immunol.* **119,** 240.

TAKAHASHI C., NISHIKAWA S., KATSURA Y. & IZUMI T. (1977) Anti-DNP antibody response after the topical application of DNFB in mice. *Immunology,* **33,** 589.

THOMAS W.R., ASHERSON G.L. & WATKINS M. (1976) Reaginic antibody produced in mice with contact sensitivity. *J. exp. Med.* **144,** 1386.

THOMAS W.R., WATKINS M.C. & ASHERSON G.L. (1978) Reaginic antibody to contact sensitizing agents. Occurrence of cells which suppress IgG and not reagin responses. *Immunology,* **35, 41.**

TURK J.L. & PARKER D. (1973) Further studies on B lymphocyte suppression in delayed hypersensitivity, indicating a possible mechanism for Jones-Motee hypersensitivity. *Immunology,* **24,** 751.

VADAS M.A., MILLER J.F.A.P., GAMBLE J. & WHITELAW A. (1975) A radioisotope method to measure delayed type hypersensitivity in the mouse. I. Studies in sensitized and normal mice. *Int. Arch. Allergy,* **49,** 670.

WOOD P., ASHERSON G.L., MAYHEW B., THOMAS W.R. & ZEMBALA M. (1977) Control of the immune reaction: T cells in immunized mice which depress the *in vivo* DNA synthesis in the lymph nodes to skin painting with the contract sensitizing agent picryl chloride. *Cell Immunol.* **30,** 25.

ZEMBALA M. & ASHERSON G.L. (1976) The effect of cyclophosphamide and irradiation on cells which suppress contact sensitivity in the mouse. *Clin. exp. Immunol.* **23,** 554.

ZEMBALA M., ASHERSON G.L., NOWOROLSKI J. & MAYHEW B. (1976) Contact sensitivity to picryl chloride: The occurrence of B suppressor cells in the lymph nodes and spleen of immunized mice. *Cell. Immunol.* **25,** 266.