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Regulation of lymphocyte proliferation in contact sensitivity: homeostatic mechanisms and a possible explanation of antigenic competition

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SUMMARY

Epicutaneous exposure of mice to the contact sensitizing chemicals 4-ethoxymethylene-2-phenyloxazol-5-one (oxazolone) and 2,4,6-trinitrochlorobenzene (picryl chloride) causes an inhibition of proliferative responses induced following subsequent topical challenge. The effects on lymphocyte proliferation comprise both transient antigen non-specific and more persistent hapten-specific mechanisms. Pretreatment of mice with one chemical 5 days prior to sensitization with a second, at which time antigen non-specific influences on proliferative responses are manifest, results in depression of contact sensitization as measured by changes in ear thickness following challenge. If, however, the period between pretreatment and sensitization is extended the inhibition of contact sensitization disappears in parallel with a decline in the antigen non-specific depression of lymph node cell proliferation. These data reveal that there exist two homeostatic mechanisms which control proliferation in response to challenge with at least some antigens, and that the extent of lymphocyte proliferation directly influences the degree of contact sensitization achieved. Moreover these results demonstrate that, in some instances at least, competition between antigens may be a function of immunoregulatory influences on lymphocyte proliferation.

INTRODUCTION

There exists compelling evidence that contact sensitization is potentially subject to a variety of immunoregulatory mechanisms, the majority of which are effected, at least in part, by suppressor T lymphocytes (Claman *et al.*, 1980a; Asherson *et al.*, 1980). Much of our understanding of the nature, and mechanism of action, of suppressor cells in contact sensitivity has been derived from studies in which such cells have been induced following the application of chemicals to animals previously exposed to UV radiation (Noonan, De Fabo & Kripke, 1981; Elmets *et al.*, 1983), by the oral administration of hapten (Asherson *et al.*, 1977) or following the intravenous injection of soluble or cell-associated hapten (Asherson & Zembala, 1974; Moorhead, 1976; Thomas, Watkins & Asherson, 1979; Miller & Claman, 1976; Miller, Sy & Claman, 1978). Such studies do not, however, directly address the question of whether the induction of sensitization following conventional topical exposure to the chemical is actively regulated.

We have previously reported that conventional epicutaneous exposure of mice to contact sensitizing chemicals such as 4-ethoxymethylene-2-phenyloxazol-5-one (oxazolone) and 2,4,6-trinitrochlorobenzene (picryl chloride) results in the appearance of a rapidly induced, systemic, suppression of subsequent proliferative responses which can be transferred to naive recipients with draining lymph node cells (Kimber *et al.*, 1987). In contrast to earlier studies in which inhibition of proliferation induced by topical exposure to chemicals was considered largely hapten-specific in nature (Asherson, Wood & Mayhew, 1975; Dunn *et al.*, 1984) our data revealed that, at least initially, the phenomenon was antigen non-specific (Kimber *et al.*, 1987). We have speculated that such regulation of proliferation is a normal homeostatic mechanism which serves to limit clonal expansion and thereby controls the vigour of the immune response (Kimber *et al.*, 1987). In the present study we report that although the inhibition of lymphocyte proliferation induced by topical exposure to chemical is initially antigen non-specific, this effect is relatively transient. The evidence indicates that exposure to a least some sensitizing chemicals results in the induction of both a short-lived hapten non-specific, and a more persistent antigen-specific, down-regulation of subsequent proliferative responses.

The data also suggest that the vigour of the primary proliferative response may directly influence the degree of contact sensitization achieved following skin painting.

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MATERIALS AND METHODS

Animals

Young adult (6–8 weeks old) BALB/c strain mice (Animal Breeding Unit, Alderley Park) were used throughout these studies.

Chemicals
4-ethoxymethylene-2-phenyloxazol-5-one (oxazolone; Sigma Chemical Co., St Louis, MO) and 2,4,6-trinitrochlorobenzene (picryl chloride; BDH, Poole, Dorset) were used as commercial preparations dissolved in 4:1 acetone: olive oil (AOO).

Measurement of lymph node cell proliferation
Untreated mice or mice which had been topically exposed to 50 μl of test chemical or vehicle (AOO) alone on the shaved flank were challenged on the dorsum of both ears with 25 μl of chemical. At various times thereafter (routinely 3 days) draining (auricular) lymph nodes were excised.

A single cell suspension of lymph node cells (LNC) was prepared under aseptic conditions by mechanical disaggregation through sterile 200-mesh stainless steel gauze. Lymphocyte suspensions were washed once in phosphate-buffered saline (PBS; pH 7·2) and resuspended in RPMI-1640 culture medium (Gibco, Paisley, Renfrewshire) supplemented with 25 mM HEPES, 400 μg/ml ampicillin, 400 μg/ml streptomycin and 10% heat-inactivated fetal calf serum (RPMI-FCS). Viable cell counts were performed by exclusion of 0·5% trypan blue and the cell concentration was adjusted to working values in RPMI-FCS. Lymphocyte suspensions were seeded into 96-well microtitre plates at a concentration of $2 \cdot 5 \times 10^6$/ml and cultured for 24 hr at 37° in a humidified atmosphere of 5% CO_2 in air with 2 μCi of [^{3}H]methyl thymidine (specific activity 2·5 Ci/mmol; Amersham International, Amersham, Bucks). Culture was terminated by automatic cell harvesting. [^{3}H]TdR incorporation was determined by β-scintillation counting.

Sensitization for and elicitation of contact sensitivity
Fifty microlitres of test chemical in AOO or an equal volume of vehicle alone were applied to the shaved flank under an occluded patch. Patches were of lint, covered by latex rubber and secured in place with a poroplast bandage (Scholl UK Ltd, London) and 1 cm tape. The patch was removed after 48 hr and 5 days following sensitization ear thickness was measured using an engineers' micrometer (Moore and Wright, Sheffield). Immediately afterwards the dorsum of both ears were treated with 25 μl of the challenge concentration of chemical. Elicitation was measured 24 hr later as the percentage increase in ear thickness.

Antigenic competition experiments
Groups of mice received 50 μl of various concentrations of the test chemical in AOO or an equal volume of AOO alone on the shaved right flank. At various times thereafter animals were sensitized on the contralateral flank with 50 μl of a second chemical under an occluded patch. Five days following sensitization, elicitation reactions were measured as described above.

In one series of experiments groups of mice were sensitized with 50 μl of 0·1% oxazolone in AOO under an occluded patch 5 days prior to treatment of the contralateral flank with 50 μl of either 1% picryl chloride in AOO or an equal volume of vehicle alone. Ten days following sensitization all mice were challenged on the dorsum of both ears with 25 μl of 0·25% oxazolone in AOO or with vehicle alone and elicitation reactions measured at 24 hr.

Table 1. Lack of antigen specificity of changes in LNC proliferation 5 days following topical exposure to oxazolone or picryl chloride

	LNC proliferation ^{3}HTdR incorporation cpm ± SD × 10^{-4} following challenge with:	
Pretreatment	1% Ox	1% Picl
2% Ox	0·89 ± 0·07	0·61 ± 0·04
1% Ox	0·93 ± 0·06	0·72 ± 0·05
0·5% Ox	1·02 ± 0·08	0·69 ± 0·05
AOO	5·43 ± 0·13	2·67 ± 1·11
2% Picl	1·04 ± 0·08	0·81 ± 0·04
1% Picl	1·32 ± 0·09	0·74 ± 0·06
0·5% Picl	1·27 ± 0·10	0·89 ± 0·07
AOO	4·79 ± 0·27	2·43 ± 0·21

Groups of mice ($n = 4$) received 50 μl of various concentrations of oxazolone (Ox), picryl chloride (PiCl) or vehicle (AOO) alone on the shaved flank. Five days later mice were challenged on the dorsum of both ears with either 1% Ox or 1% Picl. Three days following challenge the draining auricular lymph nodes were excised and a single cell suspension prepared. LNC were cultured for 24 hr at 37° in the presence of [^{3}H]methyl thymidine.

RESULTS

The antigen specificity of depression of LNC proliferation

Oxazolone and picryl chloride are immunologically non-cross-reactive. Thus, for instance, mice sensitized with oxazolone will mount an elicitation reaction following challenge with the homologous chemical, but not with picryl chloride (Kimber *et al.*, 1987).

Pretreatment of mice with various concentrations of oxazolone on the shaved flank 5 days prior to challenge of the ears with either oxazolone or picryl chloride resulted in a significant impairment of LNC proliferation compared with control animals which had received vehicle (AOO) alone (Table 1). A similar antigen non-specific depression of LNC proliferation was observed 5 days following exposure of mice to picryl chloride (Table 1).

Antigenic competition between oxazolone and picryl chloride

The lack of antigen specificity of induced changes in LNC proliferation allowed direct examination of whether pretreatment with either oxazolone or picryl chloride would influence the development of contact sensitization to the other chemical 5 days later. The hypothesis was that, if the extent of LNC proliferation is instrumental in determining the degree of sensitization then, for example, exposure to oxazolone 5 days prior to sensitization with picryl chloride should result in a reduction of the elicitation reaction following subsequent challenge with picryl chloride. This, in fact, proved to be the case, as illustrated in Fig. 1. Pre-exposure of mice to various concentrations of oxazolone caused a significant inhibition of

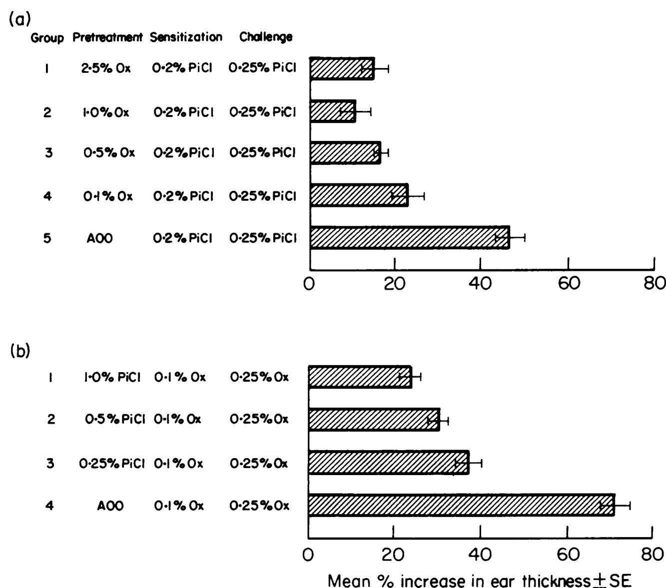

Figure 1. Antigenic competition between oxazolone and picryl chloride. Groups of mice ($n = 5$) received various concentrations of oxazolone (Ox): (a), picryl chloride (PiCl); (b), or an equal volume of vehicle (AOO) alone on the shaved right flank. Five days later mice were sensitized on the contralateral flank with 50 μl of 0·2% PiCl (a) or 0·1% Ox (b) under an occluded patch. Five days following sensitization the ear thickness of all mice was measured using an engineers' micrometer prior to challenge of the dorsum of both ears with 0·25% Picl (a) or 0·25% Ox (b). Ear thickness was re-evaluated 24 hr later and elicitation reactions recorded as the mean percentage increase in ear thickness ($\pm$SE) relative to prechallenge values.

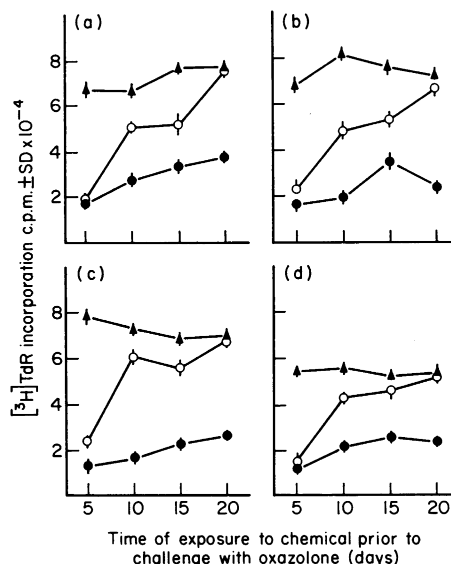

Figure 2. The persistence of induced depression of proliferative responses to oxazolone. A comparison of the effect of pre-exposure to oxazolone, picryl chloride and vehicle. Groups of mice ($n = 4$) received 50 μl of 1% oxazolone in AOO (●), 1% picryl chloride in AOO (○) or AOO alone (▲) on the shaved flank. At various times thereafter mice were challenged on the dorsum of both ears with 25 μl of 1% Ox. Three days following challenge draining auricular lymph nodes were excised. Single cell suspensions of LNC prepared under aseptic conditions were cultured for 24 hr at 37° in the presence of [^{3}H]methyl thymidine. [^{3}H]TdR incorporation is expressed as mean c.p.m. $\pm$ SD $\times 10^{-4}$. A summary of four experiments, (a)–(d).

Table 2. Failure of picryl chloride to influence elicitation reactions to oxazolone when applied following sensitization

Sensitization	Interim treatment	Challenge	Mean % increase in ear thickness ± SE
AOO	AOO	AOO	2·87 ± 3·03
AOO	AOO	Ox	7·63 ± 4·17
Ox	AOO	Ox	32·22 ± 3·79
Ox	PiCl	Ox	30·29 ± 4·28

Groups of mice ($n = 5$) were sensitized with 0·1% oxazolone (Ox) or vehicle (AOO) alone under an occluded patch. Five days later groups of mice received 50 μl of AOO or of 1% picryl chloride (PiCl) in AOO on the contralateral flank. Ten days following sensitization mice were challenged with 25 μl of either 0·25% Ox or AOO and elicitation reactions measured as described previously.

contact sensitization to picryl chloride compared to vehicle pretreated animals (Fig. 1a). Pre-exposure to picryl chloride similarly depressed contact sensitization to oxazolone (Fig. 1b).

Clearly, if the competing chemical is exerting its influence by causing down-regulation of LNC proliferation during sensitization then it would be expected that it must be given prior to sensitization. The data in Table 2 confirm this. Exposure of oxazolone-sensitized mice to picryl chloride 5 days following sensitization caused no reduction in challenged-induced elicitation reactions. It is apparent that the magnitude of the elicitation reactions detailed in Table 2 are somewhat smaller than those illustrated in Fig. 1. This is no doubt a reflection of the fact that, compared with man, contact sensitization in the mouse is relatively evanescent (Sy, Moorhead & Claman, 1979; Claman *et al.*, 1980b). In Fig. 1 the period between sensitization and challenge was 5 days, whereas in the experiments documented in Table 2 the period was extended to 10 days.

The kinetics of induced changes in LNC proliferation

In subsequent experiments the kinetics of changes induced in LNC proliferation were examined. In the first series of experiments mice received 50 μl of 1% oxazolone, 1% picryl chloride or an equal volume of AOO on the shaved flank at various periods prior to challenge of both ears with 25 μl of 1% oxazolone. The data illustrated in Fig. 2 represent the results of four independent experiments. Mice pretreated with vehicle alone exhibited maximal proliferative responses when challenged with oxazolone. As previously described, 5 days following exposure to either oxazolone or picryl chloride there was a marked inhibition of LNC proliferation in response to oxazolone. However, as the period between pre-exposure and challenge was extended (to 10, 15 or 20 days) the antigen non-specific influence on cell proliferation waned. Thus, the oxazo-

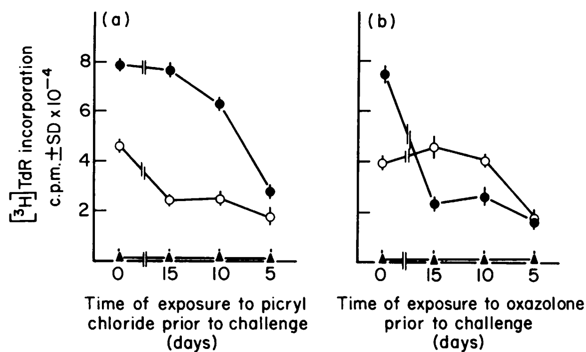

Figure 3. The persistence of hapten-induced depression of proliferative responses to 1% oxazolone (●), 1% picryl chloride (○) or vehicle (AOO) alone (▲) at various periods following exposure to (a) 1% picryl chloride or (b) 1% oxazolone. Three days following challenge auricular lymph nodes were excised and processed as previously described.

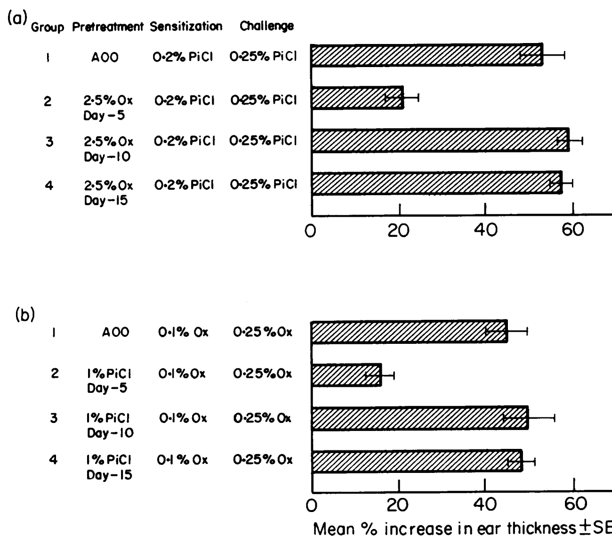

Figure 4. Persistence of antigenic competition between oxazolone and picryl chloride. Groups of mice ($n=5$) were exposed to 50 μl of 2·5% oxazolone (Ox), (a) or 1% picryl chloride (PiCl), (b) on the shaved right flank at various periods prior to sensitization on the contralateral flank with 0·2% PiCl (a) or 0·1% Ox (b) under an occluded patch. Five days following sensitization all mice were challenged on the dorsum of both ears with 25 μl of 0·25% Picl (a) or 0·25% Ox (b) and elicitation reactions measured as described previously.

lone-induced auricular LNC response in mice pretreated with picryl chloride was significantly greater than that in oxazolone-pretreated animals 10 and 15 days later. In all cases by 20 days after exposure to picryl chloride LNC proliferative responses to oxazolone were fully restored to control values. The proliferative response to oxazolone of mice pretreated 20 days earlier with the same chemical was still markedly depressed (Fig. 2). Identical patterns of reactivity were observed when pre-exposed mice were challenged at various times with picryl chloride (data not presented).

The same conclusions about the transient nature of antigen non-specific effects on LNC proliferation can be drawn from the results of experiments in which mice were pretreated with either 1% picryl chloride (Fig. 3a) or 1% oxazolone (Fig. 3b) at various periods prior to challenge with oxazolone, picryl chloride or vehicle. In both cases it is clear that while inhibition of proliferation to the homologous chemical was persistent (in these experiments for at least 15 days) the proliferative response to the second chemical was largely restored 10 days following pre-exposure and, in these studies, fully restored by 15 days. As expected, in neither case did challenge with vehicle cause any LNC proliferation.

The kinetics of antigenic competition

As the antigen non-specific influence on subsequent LNC proliferation is transient, and if, as hypothesized, the antigenic competition detailed in Fig. 1 is the result of impaired lymphocyte division, then it too should be short-lived. To examine this, experiments were performed in which mice were pre-exposed to oxazolone at various periods (5, 10 and 15 days) prior to sensitization with picryl chloride. The results illustrated in Fig. 4a demonstrate that, in accordance with the data presented in Fig. 1, sensitization to picryl chloride is suboptimal when performed 5 days following treatment with oxazolone. However, if the period between pre-exposure and sensitization is increased to 10 or 15 days then maximal sensitization to picryl chloride is achieved. An identical pattern was observed when groups of mice were pre-treated with picryl chloride at various times before sensitization to oxazolone (Fig. 4b).

DISCUSSION

The data presented reaffirm that initially the inhibition of proliferation observed following topical exposure to sensitizing agents such as oxazolone and picryl chloride is antigen non-specific in nature (Kimber *et al.*, 1987). It is clear, however, that this phenomenon is transient in as much as hapten non-specific influences on proliferation were found to have waned significantly by 10 days following primary exposure and that by 20 days responsiveness to a second antigen was fully restored. The data also reveal that, superimposed upon such short-lived hapten non-specific effects is a more persistent antigen-specific down-regulation of subsequent proliferative response to the same chemical. The co-existence of antigen-specific and non-specific mechanisms which inhibit lymphocyte proliferation may partly explain the apparent discrepancy between our previous report, in which 5 days following exposure to oxazolone or picryl chloride hapten non-specific effects were observed (Kimber *et al.*, 1987), and earlier studies in other laboratories in which down-regulation of proliferation was found to be largely, but not wholly, antigen-specific (Asherson *et al.*, 1975; Datta, Barnet & Asherson, 1976; Dunn *et al.*, 1984). Alternatively such differences may reflect variables such as the strain of mice examined.

The transient non-specific depression of proliferation induced by exposure to oxazolone or picryl chloride facilitated a direct examination of the relationship between the vigour of the proliferative response in the draining lymph node and the level of sensitization achieved. The important observation was that pre-exposure to, for instance, oxazolone substantially reduced the success of sensitization to picryl chloride 5 days later. The inference drawn is that the reduced efficiency of sensitization is

the result of impaired clonal expansion. Such a conclusion is supported by the fact that pre-exposure to oxazolone or picryl chloride influenced sensitization to the unrelated chemical only during the period when hapten non-specific depression of lymphocyte proliferation was maximal. Also compatible with this view is the demonstration that sensitization to oxazolone was unimpaired when picryl chloride was applied after, rather than prior to, exposure to oxazolone.

Taken together these data are in accordance with previous studies of antigenic competition in contact sensitivity and also provide an explanation of the mechanistic basis for this phenomenon. Thus, for instance, Wallington & Verrier Jones (1974) described inhibition of sensitization to picryl chloride in mice pre-exposed to oxazolone. In their studies maximal inhibition was observed when sensitization was performed 3–7 days following exposure to oxazolone. As the interval between pre-exposure and sensitization was extended the level of inhibition decreased and maximal elicitation reactions were fully restored by 10 days.

Although in the present study attention has focused on the action of regulatory mechanisms following conventional skin sensitization, there are several descriptions in the literature of suppressor lymphocytes induced following UV irradiation or the injection of soluble hapten which influence the afferent phase of contact allergy. It is of interest that in many instances such regulatory cells have been observed to influence lymphocyte DNA synthesis (Moorhead, 1976; Thomas *et al.*, 1979; Asherson *et al.*, 1980; Ullrich, 1985; Dieli, Abrignani & Salerno, 1987). It can be argued that the majority of afferent-acting suppressor cells influence contact sensitization through down-regulation of proliferation. This does not necessarily imply, however, that there exists only a single mechanism by which regulatory cells can modulate T-lymphocyte division. The different kinetic profiles and hapten-specificity of regulatory effects described in the present study suggest that there exist at least two cellular mechanisms through which proliferation can be influenced. It is possible that the antigen non-specific effect induced following topical exposure to chemicals which elicit a profound proliferative response in the draining lymph node is attributable to non-specific suppressor cells analogous to those described following mitogen-activation of T lymphocytes *in vitro* and which have the ability to inhibit lectin- and antigen-driven proliferation and interleukin-2 (IL-2) production (Palacios & Moller, 1981; Gullberg & Larsson, 1982; Lomnitzer, Phillips & Rabson, 1984; Carlsson, Hedlund & Sjogren, 1987; Fisher Sheehan & Swierkosz, 1987).

In the present study no attempt was made to characterize the cells which mediate hapten-specific suppression of proliferation. It is not unreasonable to suppose, however, that they are identical, or at least closely related, to afferent-acting specific suppressor cells induced following the intravenous injection of soluble hapten (Moorhead, 1976; Thomas *et al.*, 1979; Asherson, Colizzi & James, 1985; Dieli *et al.*, 1987).

Irrespective of the cellular mechanisms involved, the patterns of stimulation and subsequent regulation of proliferative responses in the draining lymph node (summarized diagrammatically in Fig. 5) have to be reconciled with the physiological requirements for control of the immune response. We speculate that hapten non-specific regulation of lymphocyte division is induced as a direct consequence of the vigorous proliferation which is elicited by some antigens. Although this effect is most

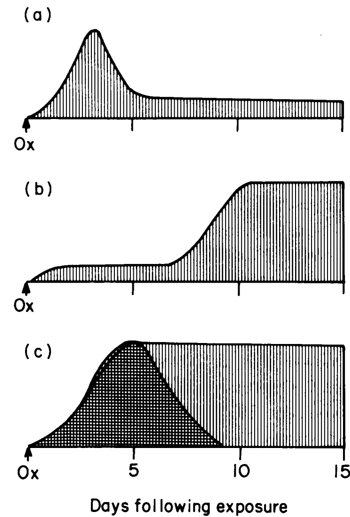

Figure 5. Diagrammatic representation of antigen-specific and antigen non-specific regulatory influences on LNC proliferation induced following primary exposure to oxazolone. (a) Responsiveness to oxazolone. Following primary exposure to oxazolone mice mount a strong proliferative response which reaches a maximum on Day 3 and then declines markedly thereafter (Kimber *et al.*, 1987). Rechallenge with oxazolone at anytime during the subsequent 20 days (at least) results in a significantly depressed proliferative response. (b) Responsiveness to picryl chloride. Primary exposure to oxazolone results in depressed proliferative responses to picryl chloride when mice are challenged 5 days later. However, within 10 days of exposure to oxazolone normal responsiveness is largely or wholly restored. (c) Suppression. A summary of regulatory influences induced by topical exposure to oxazolone. There is a short-lived, antigen non-specific depression of proliferation (vertical lines) which is superimposed upon a more persistent antigen-specific inhibition of LNC proliferation (horizontal lines).

conveniently illustrated in the context of subsequent responses to an unrelated antigen, its physiological role is undoubtedly to control the primary proliferative response to the inducing chemical and prevent excessive generation of effector cells. It may be as a consequence of this mechanism that the primary proliferative response to chemicals such as oxazolone and picryl chloride exhibit a characteristic kinetic profile wherein activity reaches a peak approximately 3 days following exposure and declines markedly thereafter (Asherson & Barnes, 1973; Asherson *et al.*, 1975; Datta *et al.*, 1976, Kimber *et al.*, 1987). Current studies are directed toward a more detailed characterization of the conditions under which hapten non-specific suppressor cells are induced and whether, as we speculate, there is a direct correlation between this phenomenon and the magnitude of the proliferative response.

Developing the argument, it is not unreasonable to suggest that the more long-lasting antigen-specific mechanism induced following conventional sensitization would provide a means of limiting proliferation following secondary exposure to the same chemical.

Although the extent to which proliferation and the development of sensitization are independently regulated is as yet unresolved, the data presented here suggest that modulation of the proliferative response in the draining lymph node may directly influence the level of sensitization achieved. The

existence of regulatory mechanisms which control antigen-driven lymphocyte division may therefore permit effective homeostasis of the immune response to skin-sensitizing chemicals and no doubt other antigens.

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