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# INHIBITION OF GUINEA-PIG LYMPHOCYTE ACTIVATION BY HISTAMINE AND HISTAMINE ANALOGUES

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**1** The incorporation of [$^3$H]-thymidine into guinea-pig lymphocytes stimulated by a plant lectin (concanavalin A), soluble antigen (tuberculin (P.P.D.)) and syngeneic hepatoma cells, was partially inhibited (50%) by histamine *in vitro*.

**2** The effect of histamine on both mitogen and antigen dose-response curves suggests a non-competitive, probably physiological antagonism.

**3** The inhibitory dose range of histamine lay between 10 nM and 30 μM with an $ID_{50}$ of approximately 400 nM.

**4** The potency order for histamine analogues for the inhibition of lymphocyte activation was histamine $\geqslant$ 4-methylhistamine > 2-methylhistamine > 3-methylhistamine. This is in accord with the mediation of the response through an $H_2$-receptor.

**5** $H_2$-receptor antagonists reversed the inhibitory effect of histamine in a dose-related manner, but both metiamide and burimamide, in high concentrations, augmented lymphocyte activation in their own right. This precluded the determination of affinity constants and made it impossible to state with certainty that the inhibition of lymphocyte activation by histamine was mediated by an $H_2$-receptor.

## Introduction

Many bacterial and viral infections induce cell-mediated reactions involving the probable proliferation of reactive thymus-derived (T) lymphocytes (W.H.O. Report 1973). This is probably a consequence of both antigen activation of sensitized cells and recruitment of unsensitized lymphocytes by secreted lymphokine factors (Dumonde, Wolstencroft, Panayi, Matthew, Morley & Howson, 1969; Wolstencroft & Dumonde, 1971). This latter process could provide an amplification system which would extend the duration and intensity of the cell-mediated lesion. The control mechanisms which might limit these reactions are not known, although several factors have been demonstrated *in vitro*. These include lymphocyte and macrophage suppressor cells (Gershon 1974), erythocytes (Yachin, 1972) and a series of local and systemic hormones: corticosteroids (Mendelsohn, Multzer & Boone, 1973), noradrenaline (Hadden, Hadden & Middleton, 1970), prostaglandin $E_2$ (Smith, Steiner & Parker, 1971; Bray, Gordon & Morley, 1976) and histamine (Plaut, Lichtenstein & Henney, 1975a, b; Rocklin 1976; Wang & Zweiman, 1978).

While these hormones may be active *in vitro*, their potential source *in vivo* at the site of cell-mediated lesions is less certain. Exceptions are prostaglandin $E_2$ synthesized by macrophages (Bray *et al.*, 1976) which infiltrate such lesions; and histamine localized in basophils which accumulate, at certain stages, in some delayed hypersensitivity reactions (Wolf-Jurgensen, 1966), the most obvious lesion being the classical Jones Mote reaction (Richerson, Dvorak & Leskowitz, 1970). Thus, a cell potentially capable of secreting a locally high concentration of histamine is a cellular feature of several cell-mediated reactions in guinea-pig and man.

We, therefore, investigated pharmacologically the effect of histamine upon lymphocyte activation in the guinea-pig, using the *in vitro* lymphocyte stimulation test. Three mitogenic stimuli were used to activate the lymphocytes: the T-cell plant mitogen, concanavalin A (Powell & Leon, 1970); the soluble antigen, tuberculin (P.P.D.); and cellular antigen, syngeneic hepatoma cells. In this way the general effect of histamine upon lymphocyte activation by a variety of immunological stimuli could be evaluated.

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## Methods

*Culture and preparation of XIII:4 hepatoma cells*

The induction and maintenance in tissue culture of the syngeneic hepatoma cell line (coded XIII:4) used in this study have been described previously (Dale, Easty, Tchao, Desai & Andjargholi, 1973). To harvest the tumour cells from monolayer culture, one part trypsin (0.25%) and two parts versene (1:5000) were incubated with the cells for 15 min. The single cell line suspension obtained was washed three times with cold culture medium and counted using trypan blue.

For sensitization of animals the cells were irradiated by X-rays for 8 min with a radiation dose of 800 rad. For lymphocyte stimulation studies the cells were 'inactivated' with 80 μg/ml of mitomycin C for 45 min. The cells were then washed four times with culture medium and recounted.

*Sensitization of guinea-pigs*

Inbred syngeneic I.C.R.F. guinea-pigs were used for sensitization with the irradiated XIII:4 hepatoma cells; $2.5 \times 10^6$ cells were injected subcutaneously into the flank of the animal. This procedure was repeated every 2 weeks for at least four immunizations. Before use in lymphocyte activation experiments, the animals were skin tested and were used only if strongly positive.

For tuberculin sensitization, normal Hartley guinea-pigs were sensitized with 1 mg of heat-killed tuberculin suspended in 0.4 ml of complete Freund's adjuvant (Difco, U.S.A.) injected subcutaneously into each hind leg and behind each ear. These animals were skin tested 3 weeks later and killed 1 week later.

*Preparation of lymphocytes*

Guinea-pigs were killed by a blow on the head and exsanguinated. The draining lymph-nodes were removed aseptically from the axillary, mesenteric and inguinal regions. Fibrous and fatty tissue was removed and the nodes gently teased apart to release the lymphocytes. The cell suspension was passed through 5 layers of surgical gauze to remove debris and cell clumps and suspended in supplemented R.P.M.I. 1640 culture medium.

The lymphocyte suspension was partially purified by spinning at 2000 *g* on a 34% triosil:9% Ficoll gradient mixed in the ratio of 10:24. The mononuclear cells (predominately lymphocytes), at the interface between the Ficoll/triosil and culture medium, were collected, washed three times, counted using a vital dye (Trypan blue) and suspended finally at a concentration of 1 to $2 \times 10^6$ lymphocytes/ml. The viability of the final lymphocyte suspension was greater than 90%.

*Lymphocyte cultures*

The procedure was based on the micro-method described by Keast & Bartholomaus (1972); 200 μl or 100 μl of the lymphocyte suspension was pipetted into 60 wells of a sterile, flat-bottomed micro-test plate (Nunc, Denmark). The outer 36 wells were filled with sterile saline and not used for culture. Tumour cells were added in 100 μl volumes, while drugs and mitogens were added in 10 μl volumes according to a randomized design.

The micro-plate was placed in a sterile, plastic incubating box containing sterile water to ensure a moist environment and incubated at 37°C in a 92% air:8% $CO_2$ atmosphere. For culture of lymphocytes stimulated by hepatoma cells, the plates were left untouched for the full incubation period, but for the studies with soluble antigen and mitogen the plates were removed after 45 min of culture and spun at 300 *g* on a Mistral 6L centrifuge (MSE). The cultures were then washed three times with culture medium before incubation. Histamine, $H_1$- and $H_2$-receptor blocking agents were maintained in culture throughout this procedure and the whole incubation; antigen and mitogen, however, were effectively removed from culture (99% removal).

After 48 h incubation 0.5 μCi of [$^3$H]-thymidine in 10 μl of sterile saline was added to each culture and the incubation continued for a further 18 h.

*Harvest of incorporated [$^3H$]-thymidine*

At the termination of the incubation the plate was spun at 350 *g* for 10 min at 3°C. The wells were then washed once with cold saline, twice with 5% trichloroacetic acid, and twice with methanol. The lymphocyte residue was then allowed to dry and finally dissolved in 100 μl of 100% formic acid. The dissolved cultures were then washed into scintillation vials (Packard Instruments) and 8 ml of scintillation fluid added. The samples were counted on a Packard scintillation counter and the ct/min converted by the automatic external standard ratio method to disintegrations per minute (d/min).

The results were expressed as the increment of incorporated [$^3$H]-thymidine as measured by d/min in stimulated cultures minus d/min in control lymphocyte cultures. Furthermore, for the hepatoma-stimulated cultures appropriate controls of tumour cells alone were subtracted from the stimulated lymphocyte counts.

*Drugs*

Concanavalin A, collagenase and diphenhydramine were obtained from Sigma U.K. Burimamide, metiamide and 2, 3 and 4-methylhistamine were donated by Smith, Kline and French U.K. Histamine acid phosphate was purchased from BDH, mepyramine maleate from May and Baker, versene from Wellcome Research, trypan blue vital dye from Gibco-Biocult and Purified Protein Derivative of tuberculin from the Ministry of Agriculture and Fisheries. All drug solutions were filtered through 0.22 μm filters (Millipore) before use.

*Reagents*

R.P.M.I. 1640 culture medium containing 40 mM L-glutamine and buffered with 25 mM HEPES (N-2 hydroxyethylpiperazine-N'-2 ethanesulphonic acid) was purchased from Gibco-Biocult. This was supplemented with penicillin, 250 units/ml (Glaxo) and streptomycin sulphate 100 μg/ml (Glaxo), 10% heat-decomplemented foetal calf serum (Gibco-Biocult) and $10^{-4}$ M aminoguanidine (Sigma).

This supplemented culture medium was used for all washing and cell cultures. Triosil 440 was obtained from Glaxo, Ficoll 400 from Phamacia, Sweden, and PPO and POPOP from Packard Instruments U.S.A. [$^3$H]-thymidine was purchased from the Radiochemical Centre, Amersham, trichloroacetic acid from Fisons, methanol and formic acid from BDH. The scintillation fluid used was a mixture of two parts of toluene containing POPOP (100 mg/litre) and PPO (5 g/litre) and one part of Triton X 100.

## Results

*Effect of histamine on mitogen and antigen dose-response curves*

Lymphocytes from guinea-pig lymph-nodes were stimulated by concanavalin A, (a non-specific T-cell mitogen), tuberculin (P.P.D.) and XIII:4 hepatoma cells. The slope of the log dose-response curves for the first two stimulants was similar, both giving bell-shaped curves. Although the explanation for the descending part of the curves is unknown, it does not appear to be due to a toxic effect of the high doses of mitogen because the cells were still viable as judged by the trypan blue exclusion test.

Figure 1 and Tables 1 and 2 show the inhibitory effect of histamine on the log dose-response curves for concanavalin A, P.P.D. and XIII:4 hepatoma cells respectively. These data illustrate that histamine partially inhibits the responses over the whole dose range of stimulant. As the curves are not shifted to the right

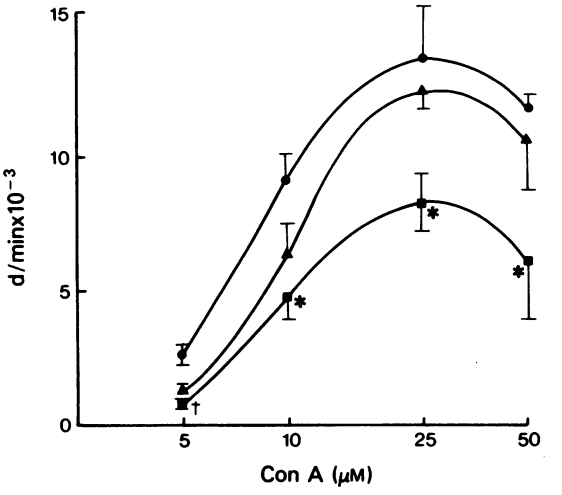


**Figure 1** Representative experiment of the inhibition by histamine of the concanavalin A-induced lymphocyte stimulation: concanavalin A (Con A) alone (●); concanavalin A + 1 μM histamine (▲); concanavalin A + 10 μM histamine (■). Each point shows the mean of five replicate cultures from a single experiment. Vertical lines indicate s.e. mean. The stimulation is expressed as the incorporation (d/min × $10^{-3}$) of [$^3$H]-thymidine per 200,000 cells. Points significantly different from control (con. A alone) using Student's $t$ test: $*P < 0.05$, $\dagger P < 0.01$.

in a parallel fashion and the maximum is depressed, it is unlikely that histamine is behaving as a competitive antagonist for mitogen or antigen receptors in this system. It is probable that the inhibition seen here is a consequence of physiological antagonism.

*Histamine dose-response curves*

The effect of histamine upon a fixed concentration of pulsed concanavalin A (12.5 μg/ml), P.P.D. (25 μg/ml) and XIII:4 hepatoma cells (40,000/ml) was tested. Figure 2 shows the dose-response curves for histamine upon these three stimuli. The inhibitory effect of histamine is clearly dose-related but the maximum effect produced by histamine is rarely greater than 50% inhibition. The dose range 10 nM to 30 μM was similar in all three experiments. The $ID_{50}$ against concanavalin A was 400 nM, against P.P.D. 300 nM, and against tumour cell stimulation 3.6 μM.

*Comparison of histamine analogues*

Four analogues of histamine were compared for their respective potency in inhibiting mitogen-induced lymphocyte stimulation. Histamine, 4, 2, and 3-methylhis-

tamine were incubated over a 1000 fold dose range with lymphocytes activated by pulsed concanavalin A (Figure 3).

The order of inhibitory potency was histamine ⩾ 4-methylhistamine > 2-methylhistamine > 3-methylhistamine. This is in accord with the known $H_2$-receptor agonist potency order of these analogues (Black, Duncan, Durant, Ganellin & Parsons, 1972). This suggests the involvement of an $H_2$-receptor in the suppression of lymphocyte activation by histamine.

*Antagonism by histamine antagonists*

In an attempt to determine the exact receptor specificity involved in this inhibition, the effect of specific $H_1$- and $H_2$-receptor antagonists was tested. In these experiments the antagonist was preincubated with the lymphocyte cultures prior to histamine addition and mitogen pulsing.

Table 3 shows the effect of two $H_1$-receptor agonists, mepyramine and diphenhydramine, tested over a 100 fold dose range. Both drugs showed some degree of antagonism of sub-maximal histamine inhibition of lymphocyte stimulation, but it is likely that these effects were non-specific. High concentrations of both blockers reduced lymphocyte activation in their own right, probably by killing lymphocytes as judged by trypan blue exclusion.

The $H_2$-receptor antagonists metiamide and burimamide (Table 4) clearly reversed the submaximal

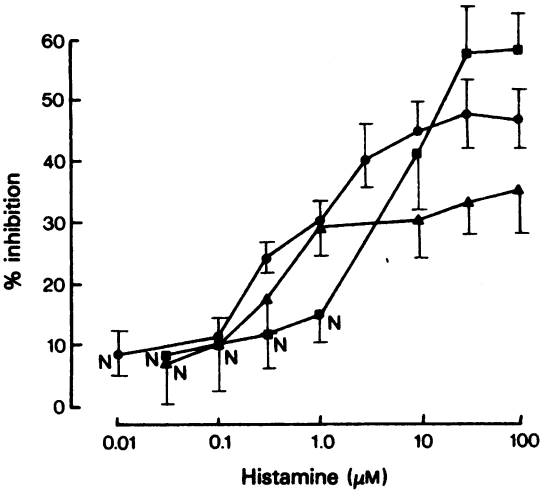


**Figure 2** The dose-related inhibition by histamine of lymphocytes stimulated by 25 μg/ml concanavalin A (●); 12 μg/ml P.P.D. (▲); 40,000 XIII:4 tumour cells/ml (■). Each point is the mean of at least 16 replicates from four experiments. Vertical lines show s.e. mean. Ordinate scale: percentage inhibition of thymidine incorporation by lymphocytes stimulated by mitogen or antigen alone. All points (except those marked N) are significantly different from conUrol (convanavalin A alone) using Student's $t$ test: $P < 0.05$; †$P < 0.01$; *$P < 0.0001$. At 100 μM 3-methylhistamine is significantly different from histamine and 4-methylhistamine: $P < 0.05$.

**Table 1** The inhibition by histamine of P.P.D.-stimulated lymphocytes

| | *P.P.D. concentration (μg/ml)* | | |
|---|---|---|---|
| | 6.25 | 12.5 | 25 |
| P.P.D. alone | 2.4 ± 0.6 | 5.2 ± 0.9 | 3.9 ± 0.9 |
| P.P.D. + 100 μM histamine | 0.9 ± 0.6 | 2.6 ± 0.6 | 1.9 ± 0.9 |
| % inhibition | 63%* | 50%* | 53% (NS) |

The stimulation is expressed as d/min × $10^{-3}$ of incorporated [$^3$H]-thymidine per 200,000 lymphocytes. Each value is the mean ± s.e. mean of 20 replicate cultures from a total of four experiments. Significant inhibition (Student's $t$ test): *$P < 0.05$. NS = not significant.

**Table 2** The inhibition by histamine of lymphocytes stimulated by syngeneic XIII:4 tumour cells

| | *XIII:4 tumour cell concentration (× $10^{-3}$/ml)* | | | |
|---|---|---|---|---|
| | 2.5 | 5 | 10 | 20 |
| XIII:4 cells alone | 5.4 ± 0.8 | 7.8 ± 1.2 | 14.5 ± 1.6 | 31.4 ± 2.4 |
| XIII:4 cells + 100 μM histamine | 3.4 ± 0.5 | 4.8 ± 0.6 | 9.3 ± 1.6 | 21.6 ± 3.1 |
| % inhibition | 37%* | 38%* | 36%* | 31%* |

The stimulation is expressed as d/min × $10^{-3}$ of incorporated [$^3$H]-thymidine per 200,000 lymphocytes. Each value is the mean ± s.e. mean of 12 replicate cultures from two experiments. Significant inhibition (Student's $t$ test): *$P < 0.05$.

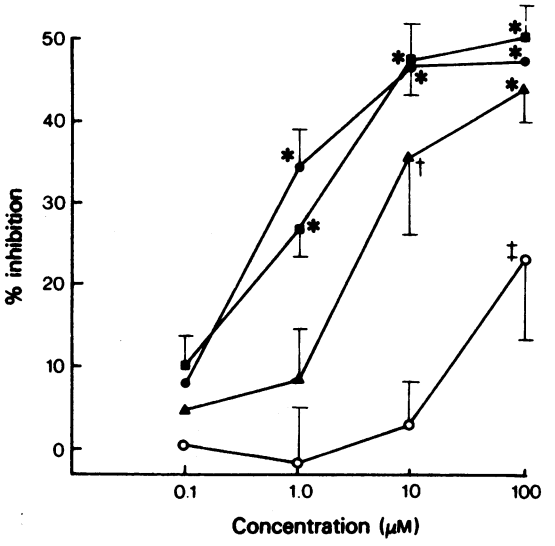


**Figure 3** The inhibition by histamine analogues of lymphocyte stimulation by convanavalin A (25 μg/ml): histamine (●); 4-methylhistamine (■); 2-methylhistamine (▲); 3-methylhistamine (○). Each point is the mean, of 12 replicate cultures from 3 experiments. Vertical lines show s.e. mean. The results are expressed as the percentage inhibition of thymidine incorporation by lymphocytes stimulated by mitogen alone. Points significantly different from control (convanavalin A alone) using Student's $t$ test: ‡$P < 0.05$; †$P < 0.01$; *$P < 0.0001$. At 100 μM 3-methylhistamine is significantly different from histamine and 4-methylhistamine: $P < 0.05$.

histamine inhibition in a dose-related manner. Both drugs achieved complete suppression between 0.1 and 1.0 μM concentrations.

These results appear to confirm the involvement of an $H_2$-receptor in the histamine inhibition of lymphocyte activation; but it must be noted that concentrations of metiamide and burimamide higher than 0.1 μM enhanced lymphocyte activation in their own right. To eliminate the possibility that $H_2$ blockers may be enhancing lymphocyte activation by antagonism of endogenous histamine, basophil counts and histamine determinations using guinea-pig ileum bioassay were made on the routine lymphocyte cultures. The average basophil count was less than 0.02% and the concentration of histamine was less than 1 ng/ml. This implies that metiamide and burimamide did not act by blocking the effects of histamine released endogenously in the cultures. The direct action of the $H_2$ blockers made it practically and theoretically difficult to demonstrate parallel shifts of the histamine log dose-response curves. Without such evidence, it is impossible to plot the dose-ratio −1 against the log dose of antagonist (Arunlaskshana & Schild, 1959) and to state definitely that histamine inhibits guinea-pig lymphocyte activation via a specific $H_2$-receptor.

## Discussion

Histamine acts through at least two specific receptors, as suggested by Ash & Schild (1966) and confirmed by Black, Duncan, Durant, Ganellin & Parsons (1972). These have been designated $H_1$- and $H_2$-receptors. In recent years much evidence has accumulated implicating the involvement of histamine, not only in immediate-type hypersensitivity, but also in cell-mediated immunity.

Histamine inhibits partially the cytolytic activity of sensitized lymphocytes, probably by increasing the intracellular cyclic adenosine 3',5'-monophosphate levels (Henney & Lichtenstein, 1971; Plaut, Lichtenstein & Henney, 1975a, b). Increasing doses of $H_2$ antagonist produced parallel shifts of the inhibitory histamine log dose-response curve (Plaut *et al.*, 1975b). Although the evidence that there was no depression of maximum responses was not clear, Schild plots were determined suggesting competitive $H_2$-receptor antagonism; the calculated affinity constants for metiamide and burimamide were close to those obtained in other known $H_2$-receptor systems. Plaut *et al.* (1975a, b) measured the cytolysis of mastocytoma cells by sensitized lymphocytes using the chromium ($^{51}Cr$) release assay (Cerottini & Brunner,

**Table 3** Effect of $H_1$ antagonists on the inhibition by histamine (5 μM) of mitogen-stimulated lymphocytes

| | *Concentration of $H_1$ antagonist (μM)* | | | | | |
|---|---|---|---|---|---|---|
| | 0 (Histamine alone) | 0.1 | 1.0 | 10 | 100 | 100 (No histamine) |
| Diphenhydramine | 52 ± 6 | 51 ± 11 (NS) | 34 ± 4* | 63 ± 4 (NS) | 94 ± 2** | 84 ± 3 |
| Mepyramine | 52 ± 6 | 39 ± 9 (NS) | 36 ± 8 (NS) | 52 ± 8 (NS) | 96 ± 1** | 89 ± 4 |

The results are expressed as % inhibition of lymphocytes stimulated by 25 μg/ml convanavalin A. Each value is the mean ± s.e. mean of 10 replicate cultures from 2 experiments. Significantly different from histamine alone: *$P < 0.05$; **$P < 0.01$ (Student's $t$ test). NS = not significant.

**Table 4** Effect of $H_2$ antagonists on the inhibition by histamine (5 $\mu$M) of mitogen-stimulated lymphocytes

| | *Concentration of $H_2$ antagonists ($\mu$M)* | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 0 (Histamine alone) | 0.001 | 0.01 | 0.1 | 1.0 | 10 | 100 | 100 (No histamine) |
| Metiamide | 27 ± 3.5 | | 12.5 ± 3** | 4.6 ± 5** | −11 ± 7‡ | −7 ± 8.5** | −21 ± 12‡ | −33 ± 7 |
| Burimamide | 23 ± 6 | 19 ± 2 (NS) | 9.5 ± 5 (NS) | 0.7 ± 7* | 0.6 ± 8* | 0 ± 9* | −7 ± 6** | −25 ± 4 |

The results are expressed as % inhibition of lymphocytes stimulated by 25 $\mu$g/ml concanavalin A. Each value is the mean ± s.e. mean of 15 replicate cultures from three experiments. Significantly different from histamine alone (Student's $t$ test): *$P < 0.05$; **$P < 0.01$; ‡$P < 0.0001$. NS = not significant.

1974). The test is believed to be a function of the killing activity of T lymphocytes (Cerottini & Brunner, 1974). It was therefore important to verify and extend these findings by measuring the actual stimulation of lymphocytes, rather than their effector function. This could confirm that any inhibition of cytolysis was a consequence of histamine acting directly upon the lymphocytes.

Rocklin (1976) and Ballet & Marler (1976) first showed that high doses of histamine could inhibit lymphocyte activation in guinea-pig and man respectively. Wang & Zweiman (1978) later reported, in a more quantitative study, the suppression of human lymphocyte activation by histamine and its reversal by metiamide but not by $H_1$ blockers. In the present investigation tests with histamine analogues suggested the involvement of an $H_2$-receptor, but conclusive confirmation using specific $H_2$ antagonists could not be obtained because of the direct augmenting action of these drugs. This enhancement did not appear to be due to the effect of endogenous histamine because no histamine could be detected in the cultures. The augmentation by $H_2$-receptor antagonists may play a role in the enhancement of skin test reactivity of cimetidine-treated patients (Avella, Binder, Madsen & Askenase, 1978). However, in the guinea-pig, metiamide did not enhance contact sensitivity (Dale, 1977).

It is nevertheless clear that histamine inhibits lymphocyte activation induced by T-cell mitogen and antigens, albeit only up to a maximum of 50%. The basophils in which histamine is localized accumulate not only in Jones Mote reactions (Richerson *et al.*, 1970) but also in lesions induced by passive transfer of lymphocytes from guinea-pigs expressing classical delayed hypersensitivity (Askenase, 1976), and in cell-mediated reactions in man induced by tuberculin, diptheria toxin and homologous lymphocytes (Wolf-Jurgensen, 1966; Dvorak, Mihm, Dvorak, Johnson, Manseau, Morgan & Colvin, 1974; Askenase, Attwood & Mangi, 1975). Furthermore, basophils and mast cells are often found at the site of animal and human tumours (Cawley & Hoch-Ligeti, 1961; Dunn, 1963; Dvorak, Dvorak & Churchill, 1973). This infiltration may be only transient, but it could play a regulatory role in any putative immunological recognition and destruction of tumours by sensitized lymphocytes.

Thus, histamine released from basophils may not only contribute to local manifestations of delayed hypersensitivity, such as erythema and increased vascular permeability, but also suppress activated lymphocytes, limiting the recruitment of further cells into the reaction site. This would then form part of a negative feedback system if the report of a histamine-releasing lymphokine can be substantiated (Theuson, Speck & Grant, 1977). Alternatively, immediate-type reactions mediated by IgE or IgG antibodies may liberate high local concentrations of histamine which may then modulate the later expression of delayed hypersensitivity (Rocklin, 1977). However, although histamine has been shown to be released at sites of delayed hypersensitivity (Askenase, Tauben & Douglas, 1977), until it has been demonstrated to be released and evoke a subsequent suppression of lymphocyte functions *in vivo*, the concept of histamine as an important modulator of cellular immunity must remain speculative.

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