Properties of a Subpopulation of T Cells Bearing Histamine Receptors

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ABSTRACT C57BL/6 mice immunized i.p. with alloantigen (P815 mastocytoma cells) develop cytolytically active thymus-derived (T) splenic lymphocytes. The definition of specific histamine receptor sites on effector T cells has been studied by measuring the in vitro effects of the hormone on cytolytic activity. Histamine was found to inhibit cytolysis reversibly and to increase lymphoid cell cyclic AMP levels. Both of these histamine activities were reversed by burimamide and metiamide; neither activity was affected by diphenhydramine or pyrilamine. These findings indicate that modulation of effector T cell activity by histamine is mediated only by one of the subtypes of tissue histamine receptors, designated a histamine-type 2 receptor. This receptor appears to be present on cytolytically active cells; there is no evidence for activation by histamine of auxiliary or "suppressor" cells.

The estimated dissociation constant (K_B) for the burimamide-receptor complex (9×10^{-6} M) and for the metiamide-receptor complex (8×10^{-7} M) indicated that the histamine receptor on T cells is quite similar to histamine-type 2 receptors in other tissues. Cells bearing such receptors could not be isolated by passage through a column of histamine-coated Sepharose beads.

The cytolytic activity of spleen cells taken from mice early (days 7–9) after immunization is virtually unaffected by histamine in vitro. In contrast, the activity of spleen cells taken from mice later in the immune response is progressively more susceptible to inhibition by histamine. After reaching a maximum at day 11, the spleen cell cytolytic activity falls in a pattern that parallels the increase in susceptibility to histamine. The susceptibility of effector T cells to histamine appears also to reflect their site of origin, for peritoneal exudate effector cells were found to be significantly less sensitive than spleen cells to inhibition by histamine.

The progressive increase in inhibition by histamine apparently reflects the appearance of greater numbers of specific histamine-type 2 receptors, and is probably a general phenomenon, for spleen cells from A/J or C3H mice immunized with either P815 mastocytoma (H-2^d) or EL-4 (H-2^b) cells showed the same effect. However, the appearance of histamine receptors could be altered by prior immunization with an unrelated alloantigen: thus, when A/J mice are preimmunized with EL-4, a subsequent immunization with mastocytoma cells results in peak spleen anti-H-2^d activity at day 9 instead of days 11–13, and the appearance of significant (> 40%) inhibition by histamine as early as day 8 instead of day 16.

The physiological role of the histamine receptors is as yet undefined, though their unexpected rate of appearance on effector T cells, coincident with a decline in the number of lytically active cells in vivo, may be a significant hint that hormone receptors play a role in the control of T-cell proliferation.

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INTRODUCTION

Recent studies on the mechanism of thymus derived (T) [1] cell-mediated cytolysis have demonstrated that the lytic activity of effector T cells can be modulated

[1] *Abbreviations used in this paper:* ATS, anti-mouse thymocyte antiserum; c, cyclic; DR, dose ratio; H, histamine; K_B, antagonist-receptor dissociation constant; MEM, Eagle's suspension culture medium; MES, medium with 10% inactivated fetal bovine serum; RSA, rabbit serum albumin; S, Sepharose; T, thymus-derived.

by a variety of exogenous hormones (1, 2). These hormones, notably prostaglandins of the E series, epinephrine, and histamine, act through pharmacologically specific receptors on the lymphocyte surface. They appear to act through a common pathway: hormone-receptor interaction activates membrane-associated adenylate cyclase. The resulting increase in cAMP in the effector cell leads to suppression of the cell's lytic activity (1–3).

Our interest in defining both the nature and role of hormone receptors on lymphoid cells has recently centered on histamine, a hormone shown to function through two types of tissue receptors. On pharmacologic grounds, Ash and Schild (4) have demonstrated that one histamine receptor (denoted "histamine-1") is antagonized by "classical" antihistamines, e.g., diphenhydramine, pyrilamine, and their analogues. It is through such sites that histamine's ability to contract bronchial or ileal smooth muscle is mediated. Histamine-2 receptor sites, on the other hand, mediate gastric acid secretion and atrial rate stimulation. These sites are unaffected by antagonists of the diphenhydramine series, but are specifically antagonized by two recently synthesized thiourea derivatives of histamine, metiamide and burimamide (5, 6).

Our preliminary studies (7) demonstrated that the histamine-induced inhibition of cytolysis was antagonized by burimamide and metiamide, but not by diphenhydramine or pyrilamine. On this basis, we proposed that effector T cells possessed only histamine-2 receptor sites. The present study represents a detailed investigation of such receptor sites on effector T cells, dealing with a quantitative definition of the site by measurements of the binding constant of antihistamine-receptor complexes. These findings revealed a striking similarity between histamine receptor sites on lymphocytes and those found on other tissues.

In other, previously reported experiments, we have shown that the effect of histamine is highly dependent on the immune status of the animal from which the effector cells are derived. When cytolytically active cells first appear (day 9), the cells are only minimally affected by histamine, but during the succeeding 7–8 days, the cells become increasingly susceptible to histamine; ultimately histamine can induce a 50% reduction in in vitro cytolytic activity (8).

The present studies detail several aspects of the developmental patterns of histamine receptors. It appears that histamine inhibition reflects two populations of T cells, one with and one without histamine receptors.

Thus, we propose that early in the immune response, the majority of cytolytically active cells lack histamine receptors. The subsequent appearance of increasing proportions of effector cells bearing histamine receptors would explain the greater ability of histamine to inhibit cytolytic activity. The increase of histamine receptors on effector cells during the immune response occurs with several strains and antigen combinations in allogeneically immunized mice. However, the relative proportion of receptors depends on the antigenic stimulus, and can be modified by prior immune stimulation. When significant proportions of effector cells with histamine receptors are present, cytolytic activity falls. This relationship suggests that the appearance of the histamine receptor is involved in control of effector cell numbers. Finally, it appears that local populations of cytolytically active cells (spleen vs. peritoneal exudate) can be defined in terms of their proportion of histamine receptor cells.

METHODS

Drugs. Histamine dihydrochloride and diphenhydramine were purchased from Sigma Chemical Co. (St. Louis, Mo.); burimamide and metiamide were the kind gift of Dr. J. W. Black (Smith Kline & French Laboratories, Herts, England). Burimamide was dissolved by warming the drug suspension at 37°C. Metiamide was dissolved in 1 M NaOH, and the solution was neutralized with HCl and stored frozen as a 0.1 M stock. Chlorpheniramine maleate was obtained from Schering Corporation (Bloomfield, N. J.), cyclizine hydrochloride from Burroughs-Wellcome Co. (Research Triangle Park, N. C.), and pyrilamine maleate from Merck & Co., Inc. (West Point, Pa.). All drug solutions were used within 2 h of preparation.

Mouse strains. C57BL/6, DBA/2, C3H/He, and A/J mice (young adult male, approximately 7–8 wk old unless stated otherwise) were obtained from Jackson Laboratories, Bar Harbor, Maine.

Splenic lymphocyte suspensions. Lymphocyte suspensions were prepared as previously described in detail (9). Briefly, the spleens were removed aseptically into Eagle's suspension culture medium containing 100 U/ml penicillin and 100 μg/ml streptomycin (MEM) and passed through wire gauze grids (mesh size 60, Small Parts, Inc., Miami, Fla.). The cells were centrifuged initially for 8 s at 800 rpm (to remove cell debris) and then washed by centrifuging three times for 5 min at 1,000 rpm in the medium containing 10% inactivated fetal bovine serum (MES). The final yield was $1.0–1.5 \times 10^8$ cells/spleen with > 90% viability as judged by trypan blue exclusion.

In some experiments, after the first wash in MEM, red blood cells were lysed by suspending the cell preparations in 0.83% NH_4Cl for 5 min at room temperature (10). In a few experiments, the erythrocyte-free preparations were further prepared by incubating the cells in glass wool columns (11) for 15 min at 37°C, eluting the nonadherent cells, and washing them once with an equal volume of MES; or by incubating the cells on Petri dishes for 30 min at 37°C, and removing the nonadherent cells with a Pasteur pipette (12).

Peritoneal exudate suspensions. Mice were killed by exposure to ether and injected i.p. with 2 ml of Eagle's medium (Microbiological Associates, Inc., Bethesda, Md.) containing 250 U heparin. Their abdomens were massaged for 60 s, and the contents removed. The peritoneal cells were then treated like the spleen cells except that they were not

passed through wire grids. The final yield was approximately 0.5×10^7 cells/peritoneum; morphologically (Wright's stain) approximately 25–40% of the cells were lymphocytes.

Histamine-coated beads. Histamine was conjugated (via 1-ethyl-3-3- dimethyl aminopropyl carbodiimide) to rabbit serum albumin (RSA); the histamine-RSA, or, as control, serum albumin alone, were attached to CNBr-activated Sepharose (S) beads (13). These beads (histamine-conjugated designated H-RSA-S, control designated RSA-S) were independently prepared in two laboratories, and were kindly provided by Drs. Yacob Weinstein, Kenneth Melmon, and Henry Bourne (University of California, San Francisco) and by Dr. Tadamitsu Kishimoto (Johns Hopkins University). The details of preparation are as previously described (13, 14).

Beads obtained from Dr. Kishimoto were tested for cell binding by a technique analogous to that described by Weinstein, Melmon, Bourne, and Sela (14). Erythrocyte-free splenic lymphocytes (3.3×10^7) plus drug were preincubated in a total volume of 0.5 ml MEM for 10 min at 37°C; then 0.5 ml of 50% H-RSA-S beads in MEM were added and incubation continued an additional 15 min at 37°C. Cells were passed through columns (as described below) and the columns washed with two 2-ml portions of MEM containing drug.

In three determinations with two nonimmune spleen cell preparations, with drug-free mixtures of cells + H-RSA-S, 48±5% (SEM) of cells applied were eluted; in the presence of 10^{-3} M diphenhydramine, 68±4% of cells were eluted; in the presence of 10^{-5} M diphenhydramine (one determination) 38% of cells were eluted.

H-RSA-Sepharose columns. 1×10^8 spleen cell suspensions in 1.2 ml MEM (no serum) were incubated with 1.2 ml of Sepharose suspensions (50% wt/vol) at 37°C for 15 min with intermittent shaking; the cell-free Sepharose mixture was then applied to a plastic column (the lower 3 ml of a 5-ml plastic pipette, with a sponge at the bottom to retain the Sepharose beads) (14), and the eluate was collected after washing with two 1-ml portions of MEM. The eluted cells were washed twice in MES, counted, and used in cytolytic assays as described below. In general, no attempts were made to recover bound cells.

Target cells (and immunization). Mouse mastocytoma cells (P815 of the DBA/2 strain) were maintained in ascitic fluid of adult DBA/2 mice. EL-4 lymphoma cells (derived from C57BL/6 mice) were maintained in ascitic fluid of adult C57BL/6 mice; both were passaged weekly. The cells were used as immunizing antigen by i.p. injection of 1×10^7 viable, washed tumor cells in 1 ml Tris buffer (pH 7.4). ^{51}Cr-labeled target cells were prepared from washed ascitic fluid; in general 10^7 cells in 0.3 ml Tris were mixed with 0.2 ml of [^{51}Cr]sodium chromate (Amersham/Searle Corp., Arlington Heights, Ill.) (diluted to approximately 0.25 mCi/ml; sp act approximately 300 μCi/μg Cr) and incubated at 37°C for 30 min; these techniques have previously been described in detail (9).

Cytolytic assays. These assays were similar to those described previously (9, 15). Immune lymphocytes were obtained from spleens of donors, generally 10–18 days after antigen administration. Lymphocytes and target cells were incubated in 1 ml MES at 37°C in a humidified atmosphere of 5% CO_2 and 95% air for up to 6 h; routinely, 10^7 lymphocytes and 10^5 target cells were incubated for 4 h. At the end of incubation, the mixture was centrifuged 5 min at 1,000 rpm (model PR-J centrifuge, Damon/IEC Div., Damon Corp., Needham Heights, Mass.), and an aliquot of the cell-free supernate was assayed for ^{51}Cr content in a γ-ray spectrometer (Nuclear-Chicago Corp., Des Plaines, Ill.).

The percentage of ^{51}Cr release was converted to percent specific release (16, 17): percent specific release = [supernate cpm (immune lymphocytes + targets) − supernate cpm (nonimmune lymphocytes + targets)]/total cpm targets, time 0.

Inhibition of specific cytolysis by drugs was calculated relative to tubes containing no drugs (2). Neither histamine nor antihistamines in the concentrations used in these experiments ever affected "spontaneous" ^{51}Cr release (i.e., ^{51}Cr release from target cells in the presence of nonimmune lymphocytes).

The range of replicate tubes containing nonimmune lymphocytes and target cells generally was < 0.5% lysis, and the range of replicate tubes containing immune lymphocyte and target cells was < 1.0% lysis. The reproducibility of the assay is demonstrated in Tables III, IX, and X.

Anti-thymocyte serum. Normal burro serum and burro anti-mouse thymocyte (ATS) antiserum were obtained from Dr. John Stobo, Mayo Clinic, Rochester, Minn.; ATS was prepared by three weekly injections with Balb/C thymocytes, as described previously (18). The specificity of this antiserum for mouse thymocytes has also been previously described (18). 2×10^7 lymphocytes were incubated in Earle's medium containing glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (Microbiological Associates) with a final concentration of 1/33 antiserum, and 1/10 guinea pig serum as complement source, for 30 min at 37°C. The cells were washed three times in MES, counted, and assayed for cytolytic activity.

Cyclic AMP assays. These were performed by mixing 1×10^7 lymphocytes from immune or nonimmune C57BL/6 mice in 1 ml MES at 37°C; 10-min incubations were used routinely because the maximum change in cAMP induced by histamine was obtained at this time. The cell suspensions were centrifuged at 1,000 rpm for 5 min, and the precipitate was resuspended in 0.5 ml 5% trichloroacetic acid. The suspension was frozen until assayed for cAMP by the method of Brown, Albano, Ekins, and Sgherzi (19), as previously adapted for the measurement of lymphocyte preparations (2, 7, 20). These measurements were kindly made by Dr. Elizabeth Gillespie, Johns Hopkins University.

RESULTS

Effect of histamine and antihistamine on cytolytic activity and cAMP levels. Histamine inhibited cytolytic activity and caused an increase in spleen cell cAMP levels. The inhibition by histamine was dose-dependent, and was maximal at 10^{-5} M; furthermore, it was almost completely reversible. Thus, after a 30–60-min preincubation of lymphocytes with histamine and one wash, the inhibition by histamine was reversed 70–95% (three experiments, data not shown).

Histamine induced increased cyclic AMP levels in whole spleen cell populations, which after 10 min of incubation were 66% above control. After 3 and 6 h the cAMP levels of histamine-stimulated spleen cells returned to base line (data not shown). Corresponding to the temporal sequence of cyclic AMP changes, the

inhibition by histamine of cytolytic activity was constant over assay periods of 1–4 h (data not shown).

Proof that the histamine receptor is pharmacologically specific requires that antihistamines specifically antagonize the histamine effect. In our preliminary report (7) we showed that the histamine-2 antagonist burimamide specifically reversed the inhibition by histamine of cytolytic activity, and also reversed the histamine-induced augmentation of cAMP levels of nonimmune cell populations. The histamine-2 antagonist metiamide also readily reverses the inhibition of cytolysis due to histamine (Fig. 1B, discussed below). On the other hand, none of the five classes of histamine-1 antagonists affected histamine's inhibition of cytolysis. Therefore, the activity of histamine is mediated through histamine-type 2 receptors.

Attempts to fractionate histamine receptor cells with histamine beads. Attempts were made to isolate the histamine-bearing cells by using histamine-coupled Sepharose beads. Weinstein et al. (13, 14, 21) and Kedar and Bonavida (22) have presented evidence that histamine, coupled through RSA to Sepharose beads (H-RSA-S) or sheep erythrocytes, binds cells that presumably have histamine receptors.

In five experiments with seven immune cell preparations, spleen cells from C57BL/6 mice immunized 12–18 days earlier were passed through an H-RSA-S columns (two such experiments are shown in Table I). The proportion of cells eluted from H-RSA-S columns were approximately 50% of those eluted from control columns (e.g., in exp. 2, 29% of the total cells eluted from the H-RSA-S column, and 66% and 59% eluted from the two control columns).

Exposure to histamine beads generally had no effect on the cytolytic activity of the cell preparations, but on one occasion (not shown) there was a twofold reduction in cytolytic activity of the H-RSA-S eluted versus control cells.

If the H-RSA-S beads are preferentially adsorbing those cells with histamine receptors, then the eluted cells should be deficient in histamine receptors; and thus histamine should have a markedly reduced effect in inhibiting the cytolytic activity of the eluted cells. However, as shown in Table I, there was no decrease in the inhibition by histamine of the H-RSA-S passed cells. Furthermore, (data not shown) the histamine receptors on the H-RSA-S-passed cells retained the same (histamine-type 2 receptor) properties of burimamide reversibility as the control cells.

Estimation of the dissociation constant (K_B) of the receptor-antihistamine complex. Since histamine-coupled beads apparently do not fractionate effector cells into a pure population of cells with histamine receptors, alternative techniques were designed to define the histamine receptor. To make quantitative comparisons of the receptor with those on other cell types, it is useful to calculate the dissociation constant, K_B, (23, 24) for the receptor-antihistamine complex. To calculate the K_B, 10^7 splenic lymphocytes obtained 13–14 days after immunization were incubated with 10^5 [^{51}Cr]mastocytoma cells in the presence of various concentrations of histamine, or with histamine together with various amounts of antagonist. Fig. 1 shows typical curves for histamine in the presence of (A) burimamide, and (B) metiamide. The dose-response curve for histamine alone was sigmoidal, with peak inhibition occurring at 10^{-5} M. In the presence of antagonist the dose-response

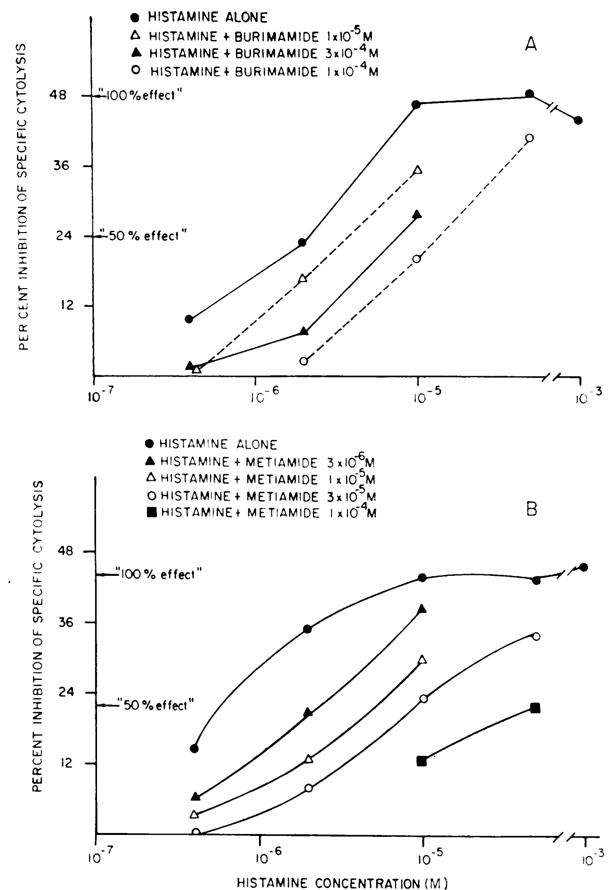

FIGURE 1 C57BL/6 mice were immunized intraperitoneally with 10^7 mastocytoma cells. After 14 days (A) or 13 days (B), spleen cell suspensions were prepared from pools of four (B) or eight (A) immune spleens. 10^7 spleen cells were incubated with 10^5 [^{51}Cr]mastocytoma cells for 4 h (A) or 3 h (B) in the presence or absence of various concentrations of histamine and either burimamide (A) or metiamide (B). Percent inhibition of specific cytolysis was calculated relative to cytolysis in drug-free culture tubes. Each point represents the mean of duplicate determinations, and cytolysis in drug-free mixtures is based on quadruplicate determinations.

Table I
*Effect of H-RSA-S Beads on the Cytolytic Activity and Inhibility by Histamine of C57BL/6 Spleen Cells**

Exp.	Days after immunization	Treatment	Recovery‡	# Cells	Specific cytolysis (no drug)	Inhibition by histamine	
						10^{-4} M	10^{-5} M
			%		%	%	%
1	12	Sp	90	2×10^6	12.5	29	32
				4×10^6	23.6	30	27
				8×10^6	39.4		27
		H-RSA-S	60	2×10^6	13.5	31	31
				4×10^6	25.1	23	27
2	13	Sp	66	1×10^6	4.4	61	44
				3×10^6	12.4	44	47
		RSA-S	59	1×10^6	3.9	61	59
				3×10^6	10.5	45	51
		H-RSA-S	29	1×10^6	4.5	65	69
				3×10^6	12.4	55	37

* C57BL/6 mice were immunized i.p. with 10^7 DBA/2 mastocytoma cells, and at intervals of either 12 (exp. 1) or 13 (exp. 2) days thereafter, spleen cells were removed and single cell suspensions prepared. Spleen cell preparations in Eagle's medium were either (treatment Sp) incubated 15 min at 37°C and transferred into plastic columns (see Methods) empty except for sponge bottom; (treatment RSA-S) incubated 15 min with RSA-S beads and then transferred into columns; or (treatment H-RSA-S) incubated 15 min with H-RSA-S beads and then transferred into columns. All assays were performed on eluted cells. Cytolytic assays were performed by incubating spleen cells and 10^5 [^{51}Cr]mastocytoma cells for 4 h in the presence of histamine. Percent specific cytolysis and percent inhibition of cytolysis by histamine were determined as in Methods. Each point is the mean of duplicate or triplicate determinations.

‡ Percentage of cells originally incubated that were eluted from the columns after final washing.

curves were shifted in parallel to the right. It is apparent that metiamide is a considerably more potent antagonist than burimamide of the histamine effect on cytolysis.

Similar data from two experiments with metiamide and two with burimamide are converted to plots of a log (dose ratio—1) vs. log (antagonist) (Fig. 2). For calculation of K_B the peak inhibition by histamine is assumed to represent 100% effect, i.e., saturation of all available histamine receptors. The dose ratio (DR) is defined as: (concentration of histamine for 50% effect, in the presence of antagonist)/(concentration of histamine for 50% effect, with no antagonist). The least square regression lines in all four experiments did not differ significantly from the theoretical slope of 1.0 if the histamine-2 antagonists were competitive inhibitors. Furthermore, the calculated K_B of approximately 9×10^{-6} M for burimamide and 8×10^{-7} M for metiamide emphasized the greater potency of metiamide. These K_B values are virtually identical to the values of K_B previously reported for histamine-type 2 receptors in guinea pig atrium (6) and human basophils (25). It thus appears that the receptor in mouse effector T cells is quantitatively similar to histamine-2 receptors in other tissues.

Evidence that cells bearing histamine receptors are effector cells. Although the preceding experiments clearly established that a histamine type-2 receptor was responsible for the inhibition by histamine of in vitro cytolytic activity, they did not define the cell (i.e., effector cell, or some type of "auxiliary" or suppressor cell) upon which the receptors appear. As the cell types cannot be fractionated by columns, the identity of the cells cannot be explored directly. We have attempted a preliminary study of the role of auxiliary cells. The three approaches we have used to suggest that histamine-receptor cells are a part of the cytolytically active cell population include depletion of adherent cells; alteration of the ratio of effector to target cells; and mixture of different effector cell populations.

Removal of adherent cells. Preliminary indications that histamine acted directly on effector cells came from the observations that the in vitro susceptibility of cytolytic activity to histamine varied during the immune response (8; see Table V). To provide more direct evidence for the role of the effector cells them-

selves (rather than some other cell) splenic cell preparations were passed through glass wool columns, and the nonadherent cells were eluted and tested for cytolytic activity and inhibition by histamine. In a typical experiment, shown in Table II, nonadherent cells had a higher cytolytic activity per 10^7 cells than the original preparation, presumably because the effector cells were more concentrated in the nonadherent population, but the nonadherent effector cells had an identical susceptibility to histamine. Similar results were obtained when cytolytically active spleen (or peritoneal exudate) cell populations were plated on Petri dishes, and the nonadherent cells assayed (data not shown). It would appear that the majority of both the histamine-receptor cells and the cytolytically active cells are part of the nonadherent (presumably lymphocyte) population.

Effect of reduction of lymphocyte/target cell ratios. If the susceptibility of effector cells to histamine depends on the relative proportions of cells with histamine receptors to cells without histamine receptors, then reduction of the number of immune lymphocytes

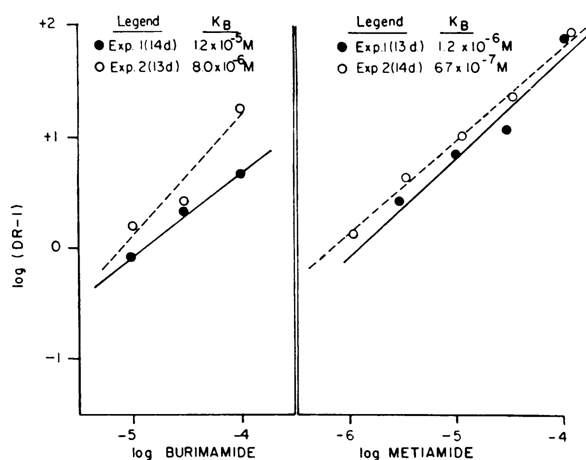

FIGURE 2 The individual points were calculated from dose-response curves of the type shown in Fig. 1A and B. For each curve, the concentration of histamine needed to produce 50% of maximal effect was estimated graphically. For each concentration of burimamide and metiamide the DR was calculated: DR = concn of hist for 50% effect in presence of antagonist/concn of histamine for 50% effect in presence of histamine alone. The linear regression lines (based on the least squares method) are shown for individual experiments. The K_B is obtained from the intersection of the line with the abscissa. The slopes and 95% confidence limits are as follows: For burimamide, exp. 1, slope = 0.77±0.21; exp. 2, slope = 1.11±0.91. For metiamide, exp. 1, slope = 0.93±0.06; exp. 2, slope = 0.89±0.66. When data from exp. 1 and 2 for burimamide are pooled, estimated $K_B = 9.4 \times 10^{-6}$ M; slope = 0.95±0.55. When data from exps. 1 and 2 for metiamide are pooled, estimated $K_B = 8.0 \times 10^{-7}$ M; slope = 0.88 ±0.16.

TABLE II

Effect of Glass Wool Columns on Cytolytic Activity and Inhibition by Histamine of Splenic Lymphocytes

		Inhibition by	
	Specific cytolysis	Histamine 10^{-5}M	Histamine 10^{-5}M +burimamide 10^{-4}M
	%	%	
Untreated	13.4	26	19
Glass wool-eluted	18.3	25	17

C57BL/6 mice were immunized i.p. with 10^7 mastocytoma cells. 13 days later, four immunized mice were killed, and single cell suspensions prepared from the pooled spleens. The cells were treated with 0.83% NH_4Cl for 5 min at room temperature to lyse erythrocytes; then washed and applied to glass wool columns for 30 min at 37°C. Cells were eluted, and columns washed with an equal volume of MES. The cells not applied to the columns (designated "untreated") and the cells eluted from glass wool were counted, and 10^7 cells of each group were incubated with 10^5 [^{51}Cr]mastocytoma cells for 4 h at 37°C. Percent specific cytolysis and percent inhibition by drugs were calculated as described in Methods. Each point is the mean of triplicate determinations.

in incubation mixtures, since it does not alter the ratio of the two types of postulated effector cells, will not alter the degree of inhibition by histamine.

If, on the other hand, the histamine receptors are present on "auxiliary" (noneffector) cells, then alteration of the lymphocyte-target cell ratio conceivably might alter histamine-induced inhibition of cytolysis (26).

In experiments designed to test this hypothesis, immune splenic lymphocytes were mixed with target cells at ratios ranging from 100:1 to 3:1. Table III shows three typical experiments. At all lymphocyte: target cell ratios, the percent inhibition of a given population of immune lymphocytes by histamine remained constant. Thus, although the role of auxiliary cells is certainly not excluded, the data are consistent with the concept that histamine receptors are on the effector cells.

Mixtures of cells. The results discussed to date could be explained either by inhibition by histamine of effector cells, or by activation by histamine of a population of what could be called "suppressor" cells. If suppressor cells are present, one might expect that the addition of cells with a large number of histamine receptors to cells with low numbers of receptors would result in a high susceptibility to histamine of the resulting mixture. To explore this question, we took advantage of the observation (8; see Table V below) of an increase in histamine receptors during the pri-

mary immune response. In six experiments, two of which are shown in Table IV, when cells with relatively low cytolytic activity and high numbers of receptors (18 days) were mixed with cells with high cytolytic activity and low numbers of histamine receptors (11 days), the resulting preparation was inhibited to a degree expected from simple addition of effector cells. In particular, despite the addition of large numbers of histamine receptor cells, in exp. 1 the cytolytic activity of 1.7×10^7 11-day cells + 8.3×10^7 18-day cells in the presence of histamine was clearly greater than the activity of 1.7×10^7 11-day cells alone + histamine. In fact, the cytolytic activity (20.0%) in this case was exactly the sum of the activities of the two populations in the presence of histamine (15.3 + 4.7). Although these "mixing" experiments cannot rule out the presence of histamine-activated suppressor cells that are relatively inefficient, the results do support the concept of two distinct effector T cell populations, one with, and one without, histamine receptors.

The pattern of histamine receptors during the primary immune response to alloantigen. To extend previous observations (8) on the effect of immunization on histamine receptors, young adult C57BL/6 mice were immunized i.p. with 10^7 mastocytoma cells, and at intervals from 7–53 days later, spleens were removed and single cell suspensions were assayed for cytolytic activity and inhibition of cytolytic activity by histamine alone or histamine and burimamide. Additionally, the effect of histamine on cAMP levels of splenic cell populations was also determined.

Table V summarizes data from over 90 experiments; some of the data is also depicted graphically in Fig. 3. In accord with our previous findings, the following points should be noted: (*a*) Cytolytic activity reached a peak 10–12 days after immunization, and then slowly

TABLE III

Effect of Variation of Lymphocyte/Target Cell Ratios on Inhibition by Histamine

	Number of lymphocytes	L/T ratio*	Specific cytolysis		Inhibition by histamine
			No drug	Histamine (10^{-5} M)	
			%		%
Exp. 1	1×10^6	10:1	7.8±0.5	7.1±0.5	9
	4×10^6	40:1	26.1±0.6	24.3±0.7	7
	7×10^6	70:1	37.1±0.8	32.9±0.3	11
	10×10^6	100:1	36.0±0.3	32.0±1.2	11
Exp. 2	3×10^5	3:1	4.6±0.1	3.7±0.5	19
	1×10^6	10:1	11.2±0.6	9.7±0.5	14
	3×10^6	30:1	32.8±0.4	25.8±0.7	21
	1×10^7	100:1	43.1±0.1	35.8±1.6	17
Exp. 3	1×10^6	10:1	6.7±0.7	4.3±0.3	35
	2×10^6	20:1	11.5±0.5	8.2±0.3	28
	5×10^6	50:1	26.9±0.5	19.3±0.8	28
	10×10^6	100:1	38.3±1.0	27.5±1.5	28

C57BL/6 mice were immunized i.p. with 10^7 mastocytoma cells. 10 days later (exp. 1), 11 days later (exp. 2), or 14 days later (exp. 3), groups of 4–5 mice were killed and suspensions prepared from pools of the spleen cells. In exps. 1 and 3, immune lymphocytes were mixed with enough nonimmune lymphocytes to make a total of 10^7 lymphocytes and incubated with 10^5 [^{51}Cr]mastocytoma cells for 4 h at 37°C. In exp. 2, the immune lymphocytes were incubated (without added nonimmune lymphocytes) with 10^5 [^{51}Cr]-mastocytoma cells, for 3 h 5 min at 37°C. In all three experiments the percent specific cytolysis and percent inhibition by histamine were calculated as described in Methods. In each experiment each value for specific cytolysis is the mean±SEM. of triplicate determinations. The percent cytolysis in the presence of nonimmune lymphocytes is (number of determinations in parenthesis) respectively: exp. 1, 15.4±2.0 (2); exp. 2, 14.0±0.2 (4); exp. 3, 13.2±0.1 (2). These values were subtracted from percent total cytolysis to yield percent specific cytolysis.

* The lymphocyte/target cell ratio (L/T ratio) is the ratio of the number of "immune" lymphocytes to target cells.

TABLE IV

Cytolytic Activity and Susceptibility to Histamine of Mixtures of Two "Immune" Spleen Cell Populations

Exp.	No. 11-day lymphocytes	No. 18-day lymphocytes	Specific cytolysis		Inhibition by histamine (10^{-5} M)
			No drug	Histamine	
			%		%
1	1.7×10^6	0	20.4	15.3	25
	1.7×10^6	8.3×10^6	28.6	20.0	30
	3.3×10^6	0	37.0	28.2	24
	3.3×10^6	8.3×10^6	39.9	29.8	26
	0	8.3×10^6	9.6	4.7	51
2	0.41×10^6	0	5.0	4.3	14
	0.41×10^6	3.3×10^6	12.0	8.0	34
	0.41×10^6	8.3×10^6	23.6	15.1	36
	0.82×10^6	0	9.7	8.0	17
	0.82×10^6	8.3×10^6	27.4	17.5	36
	0	3.3×10^6	8.5	4.7	45
	0	8.3×10^6	15.9	8.9	45

Groups of C57BL/6 animals were immunized i.p. with 10^7 DBA/2 mastocytoma cells. On the day of experiments, groups of three mice immunized 11 days earlier and three mice immunized 18 days earlier were killed, and spleen cell suspensions prepared. The indicated number of 11-day immune cells, 18-day immune cells, or mixtures of both, were mixed with nonimmune C57BL/6 splenic lymphocytes to make a total of 10^7 lymphocytes. These lymphocytes were incubated with 10^5 [^{51}Cr]mastocytoma cells for 3 h 45 min (exp. 1) or 4 h (exp. 2) at 37°C in the presence or absence of histamine. Percent specific cytolysis and percent inhibition by histamine were calculated as in Methods. In both experiments, each point is the mean of triplicate determinations.

fell. (*b*) The susceptibility of effector cells to inhibition by histamine increased markedly from day 10 to 18. (*c*) The reversal of inhibition by burimamide was essentially constant throughout the immune response. Thus, although the percentage inhibition caused by histamine may vary, the characteristics of the receptor do not change, suggesting that the proportion of effector cells with histamine receptors is increasing. (*d*) The effect of histamine on the total splenic cell population (as reflected by the percent rise in cAMP induced by histamine) did not change significantly during the immune response. (*e*) The effect of histamine on spleen cell cAMP, like the histamine effect on cytolytically active cells, is mediated through a histamine-type 2 receptor (7). Despite the changing susceptibility of effector cells to histamine, however, the cAMP levels of spleen cells were increased to the same extent during the immune response. Thus, there is no general change in the overall spleen cell histamine receptor population. Because effector cells represent $< 5\%$ of the total lymphocyte population (9), an alteration in histamine receptors in the effector cells would not be reflected in the total spleen cell pool.

Although spleen cell cytolytic activity reached a maximum on days 10–12, and then fell, there was a secondary peak of activity on approximately day 27. The secondary peak was associated with a secondary fall in susceptibility of the lymphocytes to histamine: that is, from 46% on day 18 to 32% on day 27 (Table V). This secondary fall in histamine receptors was consistently observed, and is best illustrated (Fig. 4) by a series of experiments in which a group of 50 C57BL/6 mice were immunized and tested for up to 53 days. The experiments depicted in Fig. 4 show the relationship between fall in cytolytic activity (days 10–18) and increase in histamine receptors. In these experiments the susceptibility of the lymphocytes to histamine on days 14 and 18 was clearly greater than on days 21, 24, and 27.

The increase on day 27 in cytolytic activity was associated with a decrease in histamine receptors. The data are most compatible with our hypothesis that there are two subpopulations of effector T cells (one with, and one without, histamine receptors); thus on day 27 there is apparently a reappearance of effector cells without histamine receptors.

Effect of secondary immunization. Because the increase in histamine receptors is temporally associated with a fall in cytolytic activity, attempts were made to alter the cytolytic activity and the pattern of histamine receptors by a second immunization of the C57 BL/6 mice with alloantigen. However, as illustrated in Table VI, secondary immunization on day 14 caused only moderate increases in cytolytic activity, and a boost on day 49 resulted in no significant change in cytolytic activity. In two other experiments (data not shown), secondary immunization on day 14, or day 69, resulted in no significant change in cytolytic activity when tested 4 days later. In all four experiments, cells of the secondarily immunized animals never showed significant differences in histamine receptors from those of singly immunized animals. When a second immunization was delayed until day 118, there was significantly increased activity 5 days later (2.8×10^6 lymphocytes from secondarily immunized animals killed 9.5% of 10^5 mastocytoma cells in $3\frac{1}{3}$ h, and a similar number of lymphocytes from the singly immunized animals killed 3.5% of the mastocytoma cells). 12 days after this second immunization, the cytolytic activity of lymphocytes given a second immunization had returned to base-line levels. The susceptibility to histamine on days 123–130 remained about 35%, and did not change significantly, even with a secondary response in cytolytic activity (data not shown). It was also shown that the cytolytic activity that remained on day 123 is almost certainly a function of T cells, because treatment of the effector population with burro ATS plus guinea pig complement completely abolished cytolytic activity.

TABLE V

*Cytolytic Activity and Histamine Effects during the Primary Immune Response to Alloantigens**

Days after mmunization	Specific cytolysis		Inhibition by 10^{-5} M histamine		Reversal of 10^{-5} M histamine inhibition by 10^{-4} M burimamide‖		Increase in cAMP by 10^{-5} M histamine¶	
	%	*n*	%	*n*	%	*n*	%	*n*
7	5.0±2.0§	(2)‡	7	(1)	—	—		
8	12.7±4.8	(3)	6.0±3.0	(3)	—	—		
9	30.0±6.0	(2)	10.5±1.5	(2)	—	–		
10	39.2±3.2	(10)	11.7±1.1	(12)	38.5±11.4	(9)	65, 70, 171**	
11	42.2±2.3	(19)	17.5±1.4	(22)	41.6±10.3	(9)		
12	48.8±4.6	(5)	23.4±2.3	(5)	58	(1)		
13	29.0±3.0	(17)	33.9±2.3	(17)	40.6±4.7	(8)	140	
14	25.6±2.0	(20)	36.7±2.0	(21)	51.0±5.4	(12)	52, 58	
15	22	(1)	45.5±4.5	(2)	—	–		
17	23.7±9.0	(3)	41.3±5.5	(3)	53.0±9.0	(2)	48	
18	16.0±1.8	(8)	45.5±3.0	(8)	51.0±13.0	(2)		
20	23.0±10.0	(2)	37.5±4.6	(2)	80.5±19.5	(2)		
21	16.5±4.5	(2)	41.5±1.5	(2)	48.5±0.5	(2)		
24	17	(1)	39	(1)	77	(1)		
27	28.3±2.5	(3)	31.7±3.0	(3)	57.7±0.3	(3)		
30	21	(1)	35	(1)	54	(1)		
32	34	(1)	13	(1)	47	(1)		
53	28	(1)	30	(1)	—	—		
Non-immune							66±5	(8)

* C57BL/6 mice were immunized i.p. with 10^5 mastocytoma cells. At intervals of 7–53 day later the mice were killed and spleen cell suspensions prepared. 10^7 immune spleen cells + 10^5 mastocytoma cells were incubated together for 4 h. Percent specific cytolysis and percent inhibition by histamine were calculated as in Methods. In each experiment, determinations are based on the means of duplicate to triplicate determinations. In a few (less than 10) experiments, when incubation was for periods between $3\frac{1}{4}$ and $4\frac{1}{4}$ h rather than precisely 4 h, percent specific cytolysis was adjusted to estimate value for 4 h by assuming linear killing after a 45-min lag; and in experiments where incubation was less than 3 h, percent specific cytolysis was not included in the calculations.

‡ Numbers in parenthesis refer to number of experiments.

§ Mean ±SEM.

‖ % reversal calculated as: [(% inhibition by 10^{-5} M hist) − (% inhibition by 10^{-5} M hist + 10^{-4} M Burim)]/(% inhibition by 10^{-5} M hist) × 100.

¶ 10^7 spleen cells incubated in MES with or without 10^{-5} M histamine for 10 min. Percent increase in cAMP by histamine is: [(cAMP, with hist) − (cAMP control)]/(cAMP control) × 100.

** Data from individual experiments are given.

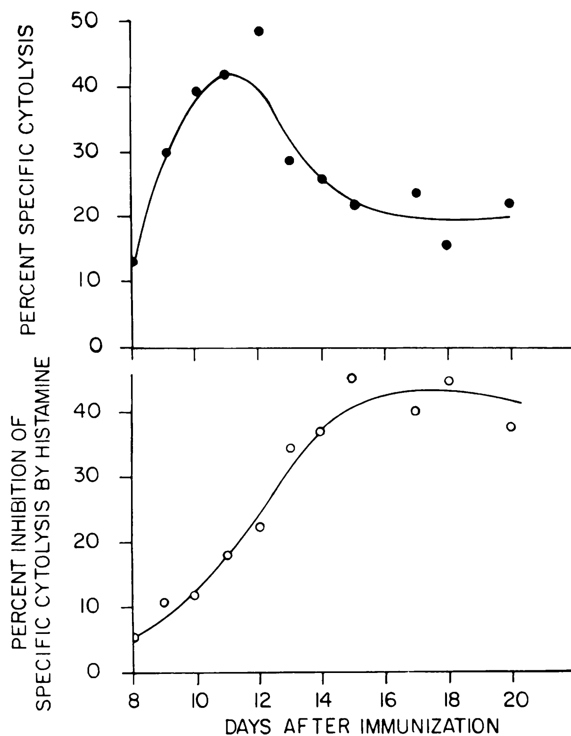

FIGURE 3 A graphic representation of data present in Table IV, for percent specific cytolysis, and for the percent inhibition of specific cytolysis by 10^{-5} M histamine, for days 8–20.

The development of histamine receptors after alloantigenic stimulation. The preceding findings have demonstrated that effector T lymphocytes from young adult (2–3 mo) C57BL/6 mice show an increase in the proportion of histamine receptors during the primary immune response. Since hormone receptors in some species may diminish during aging (27), and since cell-mediated immune responses may also be altered by aging (28, 29), the effect of aging on histamine receptors was studied in a screening experiment. Groups of 12 C57BL/6 male mice—infants 4 wk old, young adults 8 wk old, and old breeders 6 mo old—were immunized with 10^7 mastocytoma cells; and at 10 and 14 days after immunization, the mice were killed, and spleen cells examined for cytolytic activity, histamine inhibition, and cAMP levels with and without histamine stimulation.

The results (Table VII) indicate that spleen cells of all three tested ages of mice showed similar amounts of cytolytic activity. Furthermore, there was no significant difference in the pattern of histamine inhibition between the three groups; and, in all cases, the histamine inhibition was reversed approximately 40% by burimamide. The base-line cAMP values were similar in all groups, and the percent change due to histamine, although slightly smaller in the infant mice, was not significantly different. This screening experiment showed no age-associated change in histamine receptors after administration of alloantigen.

To show that the immune response-associated increase in effector cell susceptibility to histamine is a universal phenomenon after alloantigenic stimulation, the following additional systems were examined: groups of at least 15 young adult male mice, either C3H/He (H-2^k) or A/J (H-2^a), were immunized intraperitoneally with 10^7 tumor cells, either P815 mastocytoma derived from DBA/2 (H-2^d) or EL-4 lymphoma derived from C57BL/6 (H-2^b). At intervals from 8 to 28 days later, groups of three to five animals were sacrificed, and spleen cell suspensions prepared. Cells were assayed for cytolytic activity and inhibition of activity by histamine alone or histamine and burima-

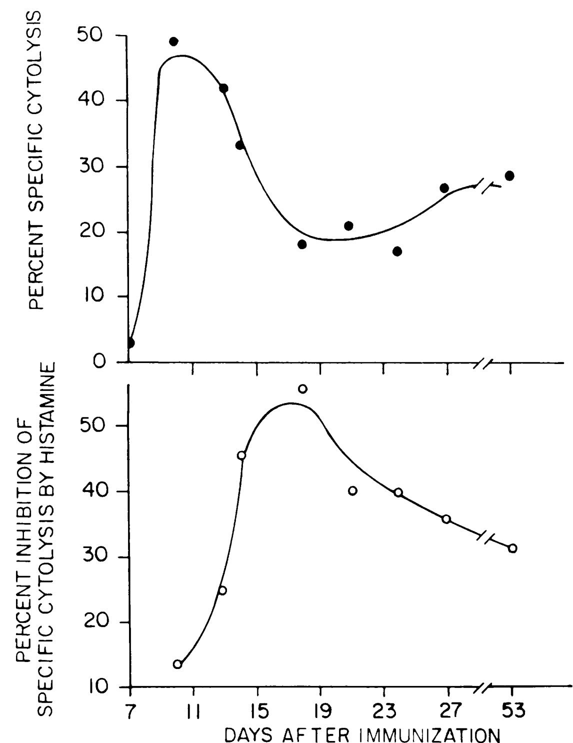

FIGURE 4 50 C57BL/6 mice were immunized intraperitoneally with 10^7 P815 mastocytoma cells. At intervals of 7–53 days later, mice were killed and spleen cell suspensions were prepared from groups of three mice. In assays, 10^7 spleen cells and 10^5 [^{51}Cr]mastocytoma cells were incubated for 4 h in the presence or absence of histamine. Percent specific cytolysis and percent inhibition were calculated as in Methods. The figure depicts inhibition in the presence of 10^{-5} M histamine, but on all days a dose-response curve for histamine from 10^{-3} M to 10^{-6} M was obtained, and the inhibition at 10^{-5} M was never significantly different from maximal inhibition. Each point is based on the mean of triplicate determinations.

TABLE VI

Effect of Secondary Immunization on Cytolytic Activity and Susceptibility to Histamine

Days after 1st immunization	Days after 2ary immunization	Specific cytolysis	Inhibition of specific cytolysis by: Histamine 10^{-4} M	Histamine 10^{-5} M	Histamine 10^{-5} M +burimamide 10^{-4} M	Histamine 10^{-5} M +metiamide 10^{-4} M
	#1	%			%	
14	—	33.4	48	46	20	7
18	—	17.9	54	58	32	28
	4	19.0	55	45	36	29
21	—	20.7	38	43	21	7
	7	28.2	30	38	13	5
24	—	16.9	39	40	9	11
	10	18.8	35	30	13	0
27	—	27.2	38	34	15	14
	13	20.3	30	26	10	4
	#2					
53	—	28.6	33	30		
	4	24.1	28	26		

50 C57BL/6 mice were immunized i.p. on day 0 with 10^7 P815 mastocytoma cells. On day 14, 12 of these "immune" mice were given a secondary immunization (#1) with 10^7 P815 mastocytoma cells. Additionally, at day 49, three mice received a secondary immunization (#2) with 10^7 P815 mastocytoma cells; these mice had previously been immunized on day 0 only. (Note that the mice immunized only on day 0 are also depicted graphically in Fig. 4). At the times indicated, pools of spleen cells from these mice were tested for cytolytic activity and inhibition of cytolysis by mixtures of histamine and either burimamide or metiamide; specific cytolysis is based on 4-h incubation of 10^7 spleen cells and 10^5 [^{51}Cr]-mastocytoma cells. Values are means of duplicate or triplicate determinations.

TABLE VII

Effect of Aging on Lymphocyte Histamine Receptors

Days after immunization	Population	Specific cytolysis	Inhibition by: 10^{-4} M Hist	10^{-5} M Hist	10^{-4} M Burimamide $+10^{-5}$ M hist	cAMP: Control	cAMP: +Hist 10^{-5} M	Change
		%		%		*pmol/10⁷ cells*		%
10	Infants	11.0	16	7	7	5.8	7.2	+23
	Young adults	20.6	18	18	11	6.5	10.7	+65
	Old breeders	10.2	20	16	10	6.8	9.8	+44
14	Infants	21.3	30	36	17	8.0	11.2	+39
	Young adults	19.5	39	46	27	6.4	10.1	+58
	Old breeders	27.2	44	41	23	6.4	9.2	+43

Groups of C57BL/6 male infants (age 3 wk), young adults (age 7 wk), or old breeders (age 6 mo) were immunized i.p. with 10^7 mastocytoma cells. On day 10 and on day 14, cell suspensions from pools of five spleens of each group were assayed by incubating 10^7 lymphocytes and 10^5 [^{51}Cr]mastocytoma cells in the presence or absence of drugs for 4 h. Percent specific cytolysis and percent inhibition of specific cytolysis by drugs were calculated as in Methods. Each value is the mean of triplicate determinations. cAMP measurements were performed by incubating 10^7 spleen cells in 1 ml MES for 10 min in the presence or absence of histamine. Each value is the mean of triplicate or quadruplicate determinations. Percent increase in cAMP was calculated as described in Table V.

TABLE VIII

Histamine Receptors in Several Inbred Mice Strains

Source of immune lymphocytes	Antigen	Exp.	Days after immunization	Specific cytolysis 4 h	Inhibition by	
					Hist 10^{-5} M	Hist 10^{-5} M +burimamide 10^{-4} M
				%	%	
C3H/He(H-2^a)	P815 (mastocytoma) (H-2^d)	1	10	3.7	16	16
			15	14.0	46	30
			18	13.9	23	10
		2	11	32.1	23	18
			14	14.7	45	29
			18	21.4	45	27
C3H/He(H-2^a)	EL-4 (lymphoma) (H-2^b)	1	8	11.3	12	12
			10	14.7	38	34
			14	11.1	28	6
		2	11	6.7	6	N.D.
			14	17.5	39	8
			18	17.0	23	12
		3	10	10.8	7	0
			15	11.8	17	6
			18	4.4	20	0
A/J(H-2^a)	EL-4 (H-2^b)	1	11	14.3	10	0
			14	11.3	35	26
			21	7.1	58	30
		2	11	12.2	12	14
			13	18.0	14	11
			14	26.9	21	13
			18	21.3	18	4
			21	18.7	31	0
A/J(H-2^a)	P815 (H-2^d)	1	11	17.4	30	26
			14	20.8	47	27
			18	9.8	56	30
			21	14.0	40	16
		2	11	30.1	23	17
			14	19.1	41	22
			18	11.1	47	25
			21	17.8	54	27
			28	15.4	53	13
		3	8	10.2	9	ND
			9	13.3	15	ND
			11	34.5	10	ND
			13	35.7	29	ND
			16	17.1	44	ND

C3H/He mice were immunized i.p. with 10^7 P815 mastocytoma or with 10^7 EL-4 cells; or A/J mice were immunized i.p. with 10^7 mastocytoma or 10^7 EL-4. At intervals noted, mice were killed and single cell suspensions were prepared from their spleens. 1×10^7 immune spleen cells were incubated in the presence or absence of drugs with 1×10^5 [^{51}Cr]target cells for 4 h, and percent specific cytolysis and inhibition by 10^{-5} M histamine or by 10^{-5} M histamine + 10^{-4} M burimamide, were calculated as described in Methods. Each point is the mean of duplicate or triplicate determinations.

mide. The results are shown in Table VIII, and some of the data are graphed in Fig. 5.

After immunization with mastocytoma cells, both C3H/He (two experiments) and A/J (three experi-

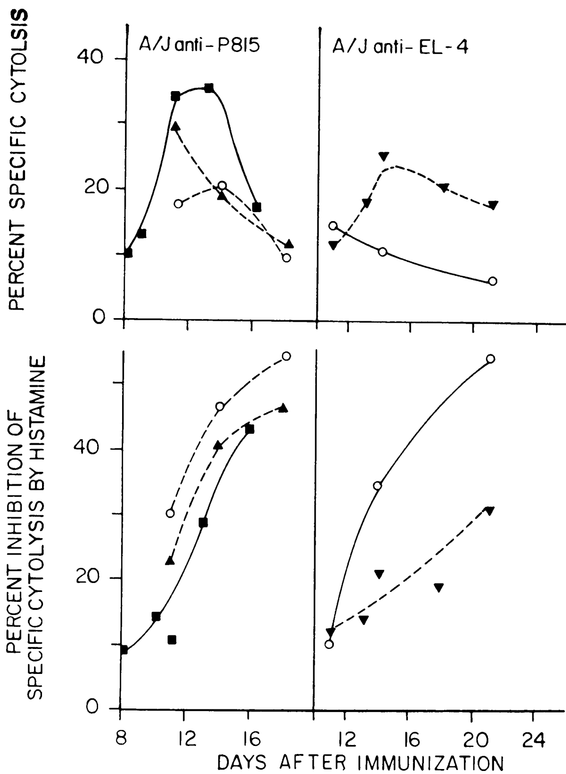

FIGURE 5 Left: Groups of A/J mice were immunized intraperitoneally with 10^7 P815 mastocytoma cells. At intervals from 8–18 days later, groups of at least three mice were killed and single cell suspensions prepared from spleens. 10^7 lymphocytes were incubated for 4 h with 10^5 [^{51}Cr]mastocytoma cells in the presence or absence of 10^{-5} M histamine. Percent specific cytolysis and percent inhibition of cytolysis was calculated as described in Methods. Each symbol (○, ▲, ■) represents a different group of animals immunized simultaneously and killed sequentially. Each value is based on the mean of triplicate determinations. Right: Groups of A/J mice were immunized intraperitoneally with 10^7 EL-4 cells, and at intervals from 11 to 21 days later groups of at least three mice were killed and single cell suspensions prepared from the spleens. 10^7 lymphocytes were incubated for 4 h with 10^5 [^{51}Cr]EL-4 cells in the presence or absence of 10^{-5} M histamine. Percent specific cytolysis and percent inhibition of cytolysis was calculated as described in Methods. Each symbol (○, ▼) represents a different group of animals immunized simultaneously and killed sequentially. Each value is based on the mean of duplicate or triplicate determinations. These graphs represent partial description of the data in Table VIII.

ments) mice developed splenic effector cells with a pattern of increasing susceptibility to histamine (Table VIII and Fig. 5) similar to that of C57BL/6 (Table V). When these strains were immunized with EL-4 lymphoma cells, effector cells appeared with a similar pattern of increased susceptibility to histamine, although the histamine receptors on the anti-EL-4 effector cells developed more slowly than in response to the P815 tumor cell antigen (Table VIII and Fig. 5). Since burimamide reversed the histamine effect in all cases, the cells appeared to demonstrate histamine-2 receptors similar to those of C57BL/6 lymphocytes.

Effect of prior immune stimulation on histamine receptors. If the pattern of susceptibility to histamine is entirely invariable, i.e., if antigenic stimuli result in the initial appearance of effector cells with low numbers of histamine receptors, followed by effector cells with increased numbers of histamine receptors, then the pattern of increasing susceptibility to histamine (Tables V and VIII) will be unaffected by prior immunization with an unrelated antigen. On the other hand, since immunization with one antigen on occasion is known to affect by "antigenic competition" (30) the immune response to a second antigen, it was considered possible that the pattern of histamine receptors might be altered by prior immunization.

To test these alternatives, groups of 25 A/J mice were initially immunized i.p. with 1.5×10^7 EL-4 lymphoma cells; 12 days later (day 0) the same mice were reimmunized with 2.5×10^7 mastocytoma cells (group I). These mice were compared to a group of 25 A/J mice immunized on day 0 with mastocytoma cells but never exposed to EL-4 cells (group II). (Immunization of mice with EL-4 cells resulted in no demonstrable lytic activity towards mastocytoma cells, and immunization with mastocytoma cells resulted in no activity towards EL-4).

The results (Table IX, which concerns cytolytic activity only against mastocytoma cells) demonstrate three significant features. First, spleens from mice preimmunized with EL-4 and subsequently immunized with P815 mastocytoma cells developed considerably fewer anti-P815 effector cells than spleens of animals immunized with P815 cells alone. Secondly, the antimastocytoma effector cells from the animals preimmunized to EL-4 demonstrated a distinct pattern of histamine susceptibility. 8 days after challenge with P815, the effector cells from group I mice were readily susceptible to histamine (> 40% inhibition), while the group II mice showed the typical pattern of increasing susceptibility up to day 16. Thirdly, in association with an early appearance of histamine receptors in the group I mice, peak cytolytic activity occurred on day 9 in the group I mice, while peak activity did not appear until days 11–13 in the group II mice.

A/J mice were immunized with EL-4 followed by P815 in four additional series of experiments. When the interval between immunization with EL-4 and immunization with P815 was 14 days, the results in two series of experiments (A and B) were similar to those in Table V, in that the peak activity of the doubly immunized animals occurred approximately on day

8, and significant (>40%) inhibition by histamine was demonstrable on day 8 (series A) or day 9 (series B). Furthermore, in both series of experiments the in vitro cytolytic activity of spleen cells from doubly immunized animals was inhibited more by histamine than activity of cells from singly immunized animals. In series A, the pattern of inhibition by histamine of effector cells from singly immunized mice was comparable to that shown in Table IX, but in series B the susceptibility to histamine developed slightly more rapidly.

When the interval between EL-4 and P815 was 7 days (series C), no anti-P815 effector cells were demonstrable, and when the interval was 8 days (series D), only minimal cytolytic activity (<2%) was present, on days 6 and 8 only, and thus inhibition by histamine could not be calculated.

In one additional experiment, where A/J mice were immunized with P815 followed by EL-4, the results were also comparable to that of Table V. The anti-EL-4 cytolytic activity in the spleen cells was lower in doubly immunized than in singly immunized mice, and susceptibility to histamine was higher in the doubly immunized mice; however, inhibition by histamine

TABLE IX

Effect of Prior Immunization on Histamine Receptors

Group	Days after immunization with mastocytoma	Number of lymphocytes	Specific cytolysis versus mastocytoma in the presence of:			Inhibition of specific cytolysis by:	
			No drug	Histamine 10^{-4} M	Histamine 10^{-5} M	Hist 10^{-4} M	Hist 10^{-5} M
				%		%	
Preimmunized	8	3.3×10^6	0.43±0.06	Not done		Not done	
"Doubly immunized"		1×10^7	2.6±0.1 (4)	1.34±0.19 (4)	0.95±0.09 (4)	48	63
Group I	9	3.3×10^6	2.6±0.1 (3)		1.2±0.1 (3)		54
		1×10^7	5.2±0.2 (3)	2.9±0.3	2.7±0.2 (3)	44	49
	11	3.3×10^6	1.4±0.3 (3)		0.72±0.11 (3)		49
		1×10^7	4.7±0.2 (3)	1.9±0.5	1.9±0.2 (3)	59	59
	13	3.3×10^6	0.54±0.07	Not calculated		Not calculated	
		1×10^7	1.7±0.1 (3)	0.40±0.27	0.95±0.15 (3)	76	44
	16	3.3×10^6	0.66±0.31	Not calculated		Not calculated	
		1×10^7	1.10±0.35	0.24±0.07	0.61±0.17 (3)	80	45
Singly immunized	8	3.3×10^6	3.5±0.2	Not done		Not done	
Group II		1×10^7	10.2±0.1 (4)	10.3±0.1	9.3±0.1 (4)	0	9
	9	3.3×10^6	5.7±0.3 (3)	4.5±0.2	4.7±0.2 (3)	20	17
		1×10^7	13.3±0.2 (3)		11.4±0.1 (3)		15
	11	3.3×10^6	15.5±0.3 (3)	14.3±0.6	13.8±0.3 (3)	7	10
		1×10^7	34.5±0.8 (3)		31.2±0.7 (3)		10
	13	3.3×10^6	18.2±0.3	14.6±0.1	13.8±0.7 (3)	20	24
		1×10^7	35.7±0.5 (3)		25.4±0.5 (3)		29
	16	3.3×10^6	10.2±0.5 (3)	6.9±0.2	6.2±0.2 (3)	32	39
		1×10^7	17.1±0.5 (3)		9.6±0.3 (3)		44

Groups of 25 A/J mice were immunized i.p. with 1.5×10^7 EL-4 cells. 12 days later ("day 0"), these animals were "doubly immunized" i.p. with 2.5×10^7 P815 mastocytoma cells. A second group of 25 A/J mice ("singly immunized") were given 2.5×10^7 mastocytoma cells on day 0, but had not been previously immunized with EL-4 cells. At intervals from 8 to 16 days later, groups of at least three mice from both the doubly and singly immunized animals were killed and single cell suspensions prepared from their spleens.

The indicated number of splenic lymphocytes (either 3.3×10^6 or 1×10^7) were incubated with 10^5 [^{51}Cr]mastocytoma cells for 4 h at 37°C in the presence or absence of 10^{-4} or 10^{-5} M histamine. Percent specific cytolysis and percent inhibition of specific cytolysis by histamine were calculated as indicated in Methods. The values represent mean±range of duplicate determinations, or mean±SEM specific cytolysis for triplicate or quadruplicate determinations. Triplicate or quadruplicate determinations are indicated by the numbers in parentheses. The values for percent cytolysis in the presence of nonimmune lymphocytes are (mean±SEM of quadruplicate determinations) respectively: day 8, 5.6±0.0; day 9, 5.9±0.0; day 11, 8.5±0.1; day 13, 6.5±0.1; day 16, 8.0±0.4.

When specific cytolysis is <2.0%, percent inhibition cannot be calculated accurately. However, when specific cytolysis in group I mice was approximately 5% (day 9 and 11), comparisons of histamine effects between group I and II mice can more readily be seen.

TABLE X

Effect of Histamine on Cytolytic Activity of Spleen Versus Peritoneal Exudate Effector Cells

Exp.	Days after immunization	Incubation time	Spleen: Effector cell/target cell ratio	Spleen: Specific cytolysis, No drug	Spleen: Specific cytolysis, Histamine 10^{-5} M	Spleen: Inhibition by histamine	Peritoneal exudate: Effector cell/target cell ratio	Peritoneal exudate: Specific cytolysis, No drug	Peritoneal exudate: Specific cytolysis, Histamine 10^{-5} M	Peritoneal exudate: Inhibition by histamine
				%		%		%		%
1	9	4 h	90:1	23.6±4.8	20.8±0.5	12	60:1	50.2±0.6	49.8±3.0	1
2	11	4 h	90:1	44.5±0.5	39.9±1.5	10	20:1	52.9±3.7	54.0±0.9	0
3	11	4 h	10:1	25.0±0.8 (3)	20.1±0.7 (3)	19	10:1	31.0±0.4 (3)	27.5±0.8 (3)	11
4	11	3 h, 5 min	30:1	32.8±0.4 (3)	25.8±0.7 (3)	21	30:1	34.0±0.7 (3)	35.5±0.8 (3)	0
5	11	3 h, 10 min	30:1	16.4±0.3 (3)	10.8±0.3 (3)	34	10:1	17.0±0.4 (3)	14.6±0.5 (3)	14
6	12	4 h	90:1	54.7±0.4	41.4±0.5	24	30:1	54.9±2.1	47.3±0.5	14
7	13	4 h	30:1	23.3±1.0	17.2±1.8	26	8:1	11.1±1.7 (3)	8.7±0.0	22
8	13	4 h	30:1	14.7±1.3	8.3±0.1	44	10:1	5.8±1.1	4.5±0.5	23
9	13	4 h	30:1	26.0±0.7 (3)	16.2±0.4 (3)	38	10:1	39.9±0.9 (3)	35.3±0.7 (3)	10
10	14	3 h, 15 min	100:1	3.9±0.4 (3)	2.5±0.4 (3)	36	30:1	11.4±0.3	9.1±0.3	20

C57BL/6 mice were immunized i.p. with 10^7 DBA/2 mastocytoma cells. Animals were killed from 9 to 14 days later, as indicated in exps. 1-10. Cell populations from pools of at least four spleens and corresponding peritoneal exudates were obtained. Various numbers of cells were incubated with 10^5 [^{51}Cr]mastocytoma cells for periods ranging from 3 h, 5 min to 4 h at 37°C. The values reported here represent from each experiment one effector cell: target cell ratio for spleen, and one for peritoneal exudate cells. (For example, 9×10^6 spleen cells: 10^5 [^{51}Cr]mastocytoma cells is an effector cell:target cell ratio of 90:1). In all ten of these experiments, additional cultures containing different lymphocyte:target cell ratios or different concentrations (5×10^{-5} M, 10^{-4} M or 10^{-3} M) of histamine yielded comparable values for percent inhibition by histamine. Percent specific cytolysis and percent inhibition of cytolysis by histamine were determined as described in Methods. The values represent mean±range of duplicate determinations, or mean ±SEM specific cytolysis for triplicate determinations. Triplicate determinations are indicated by the numbers in parentheses. The values for percent cytolysis in the presence of nonimmune, or no lymphocytes, for each experiment (mean±range of duplicate cultures, mean±SEM of triplicate or more determinations; the number of determinations is in parentheses) respectively: exp. 1, 17.1±1.5 (2); exp. 2, 7.9±0.4 (2); exp. 3, 8.2±0.2 (4); exp. 4, 14.0±0.1 (6); exp. 5, 8.2±0.6 (4); exp. 6, 6.5±0.1 (2), exp. 7, 16.5±0.5 (3); exp. 8, 9.0±0.1 (3); exp. 9, 7.3±0.0 (4); exp. 10, 10.5±0.2 (4). In these experiments, Wright's-stained preparations indicated that approximately 60-80% of spleen cells and 25-40% of peritoneal cells were morphologically lymphocytes.

reached a maximum of only 30%, and thus the differences in susceptibility to histamine between the doubly and singly immunized mice were relatively small.

Spleen versus peritoneal exudate effector cell populations. If the differential susceptibility of effector cells to histamine reflects differing proportions of two types of effector cells, lymphocytes from sites other than spleen might be expected to have somewhat different ratios of these cell populations. Groups of C57BL/6 mice were immunized with mastocytoma cells, and at intervals, from 8 to 14 days later, 3–5 mice were killed, and both spleens and peritoneal cells were removed and examined for cytolytic activity and inhibition of cytolysis by histamine. The proportion of lymphocytes was estimated by differential staining with Wright's solution; by this estimate 25–40% of peritoneal exudate cells were classified morphologically as lymphocytes. (In contrast, 60–80% of spleen cells were lymphocytes by this criterion). In some experiments, peritoneal exudate cells were fractionated by glass wool passage, and the nonadherent cells were tested. Some of the data from 10 experiments are illustrated in Table X.

In 7 of 10 experiments, peritoneal cells killed significantly more target cells than equal numbers of spleen cells. The most striking finding was that in all 10 experiments, inhibition by histamine of peritoneal effector cytolytic activity was always less than inhibition of splenic effector lymphocytes. Effector cells from the peritoneum also showed an increase with time in susceptibility to histamine. It appears that spleen cells differ from peritoneal exudate cells by possessing relatively greater proportions of effector cells bearing histamine receptors.

DISCUSSION

Histamine inhibits T lymphocyte-mediated cytolysis through interaction with a pharmacologically specific histamine-2 receptor. In attempts to characterize the cells bearing histamine receptors, spleen cell populations were passed over histamine-conjugated Sepharose beads. Although the histamine-coated beads bound a significantly greater fraction of cells than the control columns, the eluted cells were not significantly altered with respect to cytolytic activity. The eluted effector cells unexpectedly demonstrated the same susceptibility to histamine as the original spleen cell population. It would thus appear that the histamine receptors on effector cells do not bind specifically to histamine-coated beads. Since the histamine-albumin bond is via the primary amine of histamine, and because the histamine receptors (type 1 and type 2) both are presumed to bind to the primary amine group (31), it is likely that the H-RSA-S preparations have lost some of the structural specificity for histamine.

This conclusion is also supported by the recent paper of Melmon, Weinstein, Shearer, Bourne, and Bauminger (32). These authors examined histamine-induced changes in cAMP levels of mouse spleen cell populations, and compared them to spleen cells eluted from H-RSA-S beads. The cAMP levels, both control and after histamine, were lower in the H-RSA-S-excluded cells. However, the spleen cells excluded from H-RSA-S responded to histamine with a percent increase in cAMP similar to that of the unfractionated spleen cells.

In this context, it is of interest to note several reports concerning the immunological effects of histamine-coated beads: specifically, the removal of suppressor cells on H-RSA-S beads (33, 34), and similarly, termination of tolerance after removal of the cells adherent to H-RSA-S beads (35). Although the histamine beads are selectively depleting certain cell types, the present paper would strongly suggest that the binding of cells to histamine-coated beads is not through specific histamine-type 2 receptors, but because of another type of interaction (perhaps electrostatic forces, although some evidence seems to be against this interpretation (34)). We should emphasize that our assay detects a biological effect after interaction of histamine and a histamine-type 2 receptor; since we have no comparable assay for other subtypes of histamine receptor activity, we cannot at present rule out the presence of histamine-type 1 receptors. Melmon et al. (13, 14) have previously shown that histamine-1 antagonists partially block binding to H-RSA-S beads. Experiments presently in progress to assay directly histamine receptors on immune spleen cells by measurement of [^{3}H]histamine binding will provide additional information on this question.

Although we have not isolated the histamine-bearing cells, we have suggested by several approaches that the histamine-mediated inhibition of cytolysis is through receptors on the cytolytically active effector cells. Thus, alteration of lymphocyte to target cell ratios did not affect quantitatively the histamine-induced inhibition of cytolysis; and mixtures of different effector cell populations were additive. Both of these findings are in keeping with the hypothesis that the histamine receptors are on a subpopulation of effector cells. We cannot entirely exclude the importance of noneffector cells or even suppressor cells.

The evidence that the effect being measured is mediated by specific histamine receptors is compelling. The capacity of reversible competitive antagonists to alter the sensitivity of a tissue to an antagonist can be quantitated by determining the apparent dissociation constant, K_B, of the receptor-antagonist complex. If K_B values for a given competitive antagonist are similar, then the receptors may be assumed to be similar (24). Since the inhibition of cytolysis by combinations of histamine and burimamide, or metiamide, are similar at 1, 2, or 4 h (data not shown), conditions after 3–4 h incubation approximate equilibrium, and thus K_B's can be calculated for metiamide and burimamide. The slopes of log $(\mathrm{DR}-1)$ vs. log (antagonist) are close to 1, and thus confirm that these are competitive antagonists. The calculated K_B values of 9×10^{-6} M for burimamide compare to values in guinea pig atrium of 8×10^{-6} M, rat uterus of 7×10^{-6} M (6), and human basophils of $4\text{–}5 \times 10^{-6}$ M (25), and similarly, the value of 8×10^{-7} M for metiamide compares to the value in guinea pig atrium of 9×10^{-7} M, rat uterus of 8×10^{-7} M (6), and human basophils $3\text{–}8 \times 10^{-7}$ M (25). We have thus defined a receptor on mouse lymphocytes that is very similar to the histamine-2 receptors on other tissues.

It is apparent that intraperitoneal immunization with allogeneic tumor cells results in cytolytically active splenic lymphocytes which become progressively more susceptible to in vitro inhibition by histamine. This pattern has been confirmed in over a hundred experiments employing several different alloantigens and is present in mice of several ages.

The changing susceptibility of lymphocytes to histamine during the primary immune response confirms that the lymphocyte population and not the target cell is being affected by the drug. We interpret the altered susceptibility to histamine as being due to an increase in histamine-type 2 receptors. Several alternative explanations do, however, deserve consideration. Two of these, altered affinity of histamine receptors and altered histamine metabolism, seem unlikely. At all times after immunization, (*a*) the dose-response curves for histamine are similar (maximal inhibition at 10^{-5} M concentration) (8); (*b*) endogenous histamine release from suspensions of splenic lymphocytes and mastocytoma cells is insignificant during the in vitro assay, and exogenous histamine is not significantly reduced during the assay (data not shown); and (*c*) the reversal by burimamide of histamine inhibition is quantitatively identical (8, and Table V).

Evaluation of a third possibility, namely that nonfunctional receptor-adenylate cyclase complexes become functional, awaits direct measurements of receptors on effector cells.

The emergence of hormone receptors has previously been shown to occur during the in vitro differentiation of several tissues (36), including lymphocytes (37). Thus, the histamine receptors could arise during the late stages of differentiation of effector cells; and the pattern of appearance of receptors could be due to the combined effects of differentiation, death, and migration patterns. Alternatively, the increasing inhibition

by histamine could represent the appearance during the immune response of a distinct subpopulation of effector cells bearing histamine receptors, which increases in number relative to effector cells lacking histamine receptors. The data cannot distinguish between these two possibilities. Experiments that demonstrate that late in the immune response (days 21–27 versus day 18, Table VI and Fig. 4), spleen cells have increased cytolytic activity and decreased susceptibility to histamine would be more in keeping with the hypothesis that there are two distinct subpopulations of T effector cells.

From the data in Tables V and VIII, it would appear that the increase in histamine-type 2 receptors during the primary immune response to alloantigen is common to several mouse strains immunized with several antigens. The stimulus to the increase in susceptibility to histamine is not known. In an attempt to explore the antigenic specificity of this stimulus, experiments of the type shown in Table IX were undertaken. The P815 ($H\text{-}2^d$) and EL-4 ($H\text{-}2^b$) tumor cells were chosen because no cross-reacting H-2 specificities in these antigens are recognized by A/J mice (38, 39). A similar approach has been described with another combination of two tumor cell lines (40). It is conceivable that non-H-2 antigens or tumor or virus-specific antigens may induce cross-reactivity. Furthermore, recent evidence suggests that the cytolytically active lymphocytes may be directed to specificities distinct from serologic H-2 specificities (41). However, since spleen cells of A/J mice immunized against EL-4 never lysed P815 mastocytoma cells, and conversely, spleen cells of A/J mice immunized against P815 never lysed EL-4 cells, it would appear that these tumor cells are not significantly cross-reactive under these conditions.

It is apparent that the cytolytic activity of spleen cells of preimmunized mice can be inhibited by more than 40% as early as 8 days after immunization with mastocytoma cells; this pattern is quite distinct from the cytolytically active spleen cells of singly immunized mice, which are inhibited $> 30\%$ by histamine only 16 days after immunization.

The data in Table IX also demonstrate a phenomenon analogous to antigenic competition (30); the cytolytic activity against mastocytoma cells is considerably reduced by prior immunization with EL-4; similar results were obtained when mastocytoma cells were the first antigen and EL-4 the second antigen (see Results). The mechanisms(s) of antigenic competition are not clear; the splenomegaly induced by the initial antigen may explain the effect (42), but competition for specific cell types or T cell-inhibitory substances may be important (30, 43, 44). If, as we have postulated, histamine receptors belong to a distinct subpopulation of effector T lymphocytes, then prior immunization has altered not only the number of cytolytically active T lymphocytes, but also the proportions of those cells with histamine receptors. Alternative methods of inducing cytolytically active cells may also alter the numbers of cells that bear histamine receptors. For example, graft-versus-host reactions in X-irradiated DBA/2 animals reconstituted with either normal or immune C57BL/6 spleen cells result in cytolytically active P815 cells that are virtually unaffected ($< 5\%$ inhibition) by histamine.[2] Therefore, the pattern of appearance of histamine receptors during the immune response depends upon the method of induction of cytolytically active cells.

After i.p. immunization the increase in histamine receptors on effector cells is a highly reproducible phenomenon. To define regional differences in cytolytically active cell populations, we have compared peritoneal exudate to spleen cells. The results (Table X) confirm previous reports that immune peritoneal exudate cells have higher cytolytic activity than corresponding numbers of spleen cells (45–48), but in addition the experiments demonstrate a significantly lower susceptibility to histamine in exudates than in corresponding spleen cells. These differences may permit use of histamine susceptibility as a marker for regional populations of effector cells.

We must consider two as yet unsolved problems. First, the properties of the cell subpopulations that mediate the histamine effect remain unknown. It is known that, during the course of the primary immune response to alloantigen, cytolytically active cells, although heterogeneously distributed on density gradients, tend to become smaller and denser (49–52); these changes may represent either stages of differentiation or distinct populations of effector cells. In further attempts to characterize the histamine receptor-bearing cells, we have recently begun to fractionate spleen cell populations on Ficoll gradients (52). By this method we can demonstrate that certain fractions are enriched while others are depleted in their susceptibility to inhibition by histamine, but that the histamine receptors are present on both small and large lymphocytes.[3] We thus have more direct evidence that histamine affects only a subpopulation of the cytolytically active cells. However, we have not been able to separate completely the histamine-receptor bearing cells.

The second problem concerns the significance of the increase of histamine receptors. Several groups of investigators have noted increases in histamine and in histamine decarboxylase activity during allograft rejection (53–55), and have suggested that histamine

[2] Plaut, M., and C. S. Henney. Unpublished observations.
[3] Plaut, M. Unpublished observations.

decarboxylase is physiologically important in allograft rejection (56), but we have not as yet measured histamine decarboxylase in our system. We have noted that there characteristically is an inverse relationship between cytolytic activity and histamine susceptibility. When cells are inhibited 30–40% or more by histamine, the cytolytic activity falls (Tables V and VIII, Fig. 3); in the doubly immunized animals (Table IX, group I), the high degree of histamine-induced inhibition of cytolysis on day 8 is associated with an unusually early fall in cytolytic activity (after day 9). It is conceivable that histamine, through its receptor, may have a role in the control of T cell proliferation.

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