Separation of Specific Antibody-Forming Mouse Cells by their Adherence to Insolubilized Endogenous Hormones

KENNETH L. MELMON, YACOB WEINSTEIN, G. M. SHEARER, HENRY R. BOURNE, and SARA BAUMINGER

From the Division of Clinical Pharmacology, Departments of Medicine and Pharmacology, University of California Medical Center, San Francisco, California 94143, and the Departments of Chemical Immunology and Biodynamics, The Weizmann Institute of Science, Rehovot, Israel

ABSTRACT Spleen cells from mice immunized with sheep red cells were separated by differential adherence to insolubilized histamine, catecholamines, and prostaglandins. The hormones were insolubilized by linking them to Sepharose beads through a protein carrier. We measured hemolytic plaque formation (per million splenic leukocytes) of cells which passed through columns of hormone-carrier-Sepharose beads (i.e., those cells that failed to bind). As compared with control (no column) cells, the number of plaque-forming cells was substantially reduced by passage through histamine, epinephrine, isoproterenol, and prostaglandin-E_2 columns. Plaque-forming cells were not significantly reduced by passage through carrier Sepharose (another control) or norepinephrine- and prostaglandin-$F_{2\alpha}$-carrier Sepharose columns. Thus, the ability of an insolubilized hormone preparation to subtract plaque-forming cells roughly correlated with the presence of pharmacologic receptors for the corresponding free hormones, as judged by stimulation of cyclic AMP accumulation in the same cells, reported previously. Both 19S and 7S plaque-forming cells were subtracted by columns prepared from pharmacologically active hormones, but none of the insolubilized hormones stimulated accumulation of intracellular cyclic AMP. The cell membrane phenomenon that allows adherence to a given hormone–carrier-bead column may be identical with the cell receptor.

INTRODUCTION

Recent studies have shown that small portions of haptens or whole antibodies can be specifically recognized by receptors on a variety of immunocompetent cells (1–5). In some of these studies, the hapten or the antigen was insolubilized by covalent linkage to beads of glass, nylon, polyacrylamide, or agarose (Sepharose) (6–9). Columns of antigen-Sepharose allowed the cells with receptors to be bound to the immunologic attractant while other cells without receptors passed through the column (6, 7). We have recently used essentially the same techniques to demonstrate that human peripheral and mouse spleen leukocytes could be separated by batch chromatography on columns made of biogenic amines and prostaglandins linked to protein or polymer carriers and insolubilized by covalent linkage to Sepharose beads. Separation of the cells, at least in part, seemed to be related to their physiologic receptors for the insolubilized hormone (10, 11).

A number of studies have demonstrated pharmacologic receptors on leukocytes for histamine, beta-adrenergic catecholamines, and the E-series prostaglandins (PGE)[1] (12–15). Stimulation of these discrete receptors results in activation of adenyl cyclase and accumulation of cyclic AMP within the cell. Possibly because of increased production of cyclic 3′,5′-AMP, there is inhibition of IgE-mediated release of histamine and other mediators of inflammation from human basophils, human and monkey lung, and rat mast cells (14, 16–20), inhibition of immunologically specific cytolytic activity

Dr. Shearer's present address is: Immunology Branch, National Cancer Institute, National Institutes of Health, Bethesda, Md. 20014.

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[1] *Abbreviations used in this paper:* ECDI, 1-ethyl-3-3 dimethyl aminopropyl carbodiimide; MEM, minimal essential medium; NE(GA)-P-S, norepinephrine linked via glutaraldehyde to polymer and then to Sepharose; NE (GA)-RSA-S, norepinephrine linked via glutaraldehyde to RSA and then to Sepharose; NE-RSA-S, norepinephrine bound via ECDI to RSA and Sepharose; P, polymer of DL-alanine-L-tyrosine, mol wt, 1,800; PBS, 0.15 M NaCl in 0.01 M sodium phosphate pH 7.4; PFC, plaque-forming cells; PG, prostaglandin; RSA, rabbit serum albumin; SRC, sheep red blood cells.

of mouse splenic lymphocytes (15, 20, 21), and inhibition of release of antibody from leukocytes in vitro (22).

We have studied some properties of immunocompetent cells with "receptors" for endogenous hormones by subtracting them from other splenic leukocytes with insolubilized histamine columns. We found that mouse spleen leukocytes expressing histamine receptors could regulate the magnitude of plaque-forming cell (PFC) response in an adoptive cell transfer (23). In the present study, a second immunologic function, the release of antibody from lymphocytes, has been assessed by hemolytic plaque formation in agar. The results indicate that a substantial portion of the splenic cells that produce antibodies to sheep red cells (SRC) can be subtracted from the total spleen leukocyte population by columns of insolubilized histamine, beta-catecholamines and the E-series prostaglandins. The PFC are not subtracted by columns made of insolubilized hormones for which there do not seem to be corresponding pharmacologic receptors.

METHODS

Three strains of mice [C57BL/6, BALB/c, and (BALB/c × C57BL/6) F_1 = BALB/BL] were supplied by the animal breeding house of the Weizmann Institute. The mice were immunized by intraperitoneal injection of 3×10^8 SRC and spleens were removed on day 6, 9, or 13 after immunization. The cells were teased from the spleens, strained through stainless steel mesh (200 mesh), and suspended in Eagle's minimal essential media (MEM), pH 7.4 (Microbiological Associates, Jerusalem). The hemolytic plaque assays were done by a modification of the Jerne, Nordin, and Henry agar plate technique (24, 25).

The assays were performed in agarose (L'Industrie Biologique Française S.A., Gennevilliers, France) on 60-mm plastic disposable Petri dishes (25). The plates were incubated at 37° for 1 h, and then for an additional h in the presence of 1 ml of 1:10 diluted lyophilized guinea pig complement (Grand Island Biological Co., Grand Island, N. Y.). Direct PFC were counted after the 2-h incubation period. Control plates contained between 80 and 150 plaques/plate. Indirect PFC were developed on the same plates (after the direct PFC had been marked) by incubation for 2 additional h in the presence of 0.1 ml 1:100 diluted rabbit anti-mouse IgG (26). Triplicate plates were made from each sample and read by two investigators at different times. Rosette formation was assayed by the method of Shearer and Cudkowicz (27).

Preparation of hormone-Sepharose. Separate batches of histamine, isoproterenol, and PGE_1, E_2 and $F_{2\alpha}$ were conjugated either to rabbit serum albumin (RSA) or to a synthetic random copolymer (P) of DL-alanine-L-tyrosine (average mol wt of 1,800), by incubating their solutions with 1-ethyl-3-3 dimethyl aminopropyl carbodiimide (ECDI) by methods previously described (10, 11). The resultant conjugate, RSA, or polymer alone was attached to Sepharose that had been activated by CNBr by the method of Porath, Axen, and Ernback (28). The details of each preparation and the characterization of the resultant hormone-RSA-Sepharose have been reported elsewhere (11).

Norepinephrine was also conjugated either to RSA-Sepharose or polymer (P)-Sepharose via glutaraldehyde and later attached to Sepharose [NE(GA)-R-S or NE (GA)-P-S] by the method of Weinstein, Melmon, Bourne, and Sela (11). Such a process converted the norepinephrine into a substance whose qualities resembled insolubilized epinephrine. The Sepharose preparations were stored as a 25% (wt/vol) suspension in 0.15 M NaCl in 0.01 M sodium phosphate pH 7.4 (PBS) in the cold until used, at which time they were washed with 50 vol of MEM.

Cell incubates and hormone-RSA-Sepharose columns. The Sepharose preparations, 0.6 ml of a 25% (wt/vol) of hormone-RSA-Sepharose in MEM, were incubated in plastic tubes with $10–200 \times 10^6$ spleen cells at 37°C for 15 min with gentle intermittent shaking. The cell-Sepharose mixture was then poured into a plastic column and the unbound cells collected after the column was washed with two 1-ml aliquots of MEM (11). Control preparations of cells were made with RSA-Sepharose, activated Sepharose, or Sepharose alone. Equal numbers of control and experimental cells were then used for the rosette or plaque assay with SRC. More than 90% of the cells that passed through the columns remained viable, as assessed by exclusion of Trypan blue. This percentage did not change, regardless of the column material used. No studies on the morphology of cells excluded by various columns have been attempted.

The ability of insolubilized hormone preparations to stimulate accumulation of intracellular cyclic AMP was tested. Incubation of 50×10^6 cells with 0.6 ml (25% wt/vol) of one of the Sepharose preparations and theophylline, 1×10^{-2} M, was carried out for 30 min at 37°C with gentle shaking. Then the supernate was discarded and the cells assayed for cyclic AMP content. The content of cyclic AMP in the supernate was not measured, though such studies are anticipated. By the criterion of intracellular accumulation of cyclic AMP, there were two pharmacologically active preparations, PGE_2-P-S and NE(GA)-P-S. The supernates from the stored Sepharose preparation of NE(GA)-P-S as well as the supernate from the Sepharose-plus-cell incubate were reincubated in the same conditions with fresh batches of cells to determine whether sufficient hormone or hormone conjugate had been released spontaneously or by the action of cells to stimulate other cells. Cell supernates were found to be inactive, by the criterion of intracellular cyclic AMP accumulation.

Assay of leukocyte cyclic AMP. The cyclic AMP was measured in duplicate samples each containing 10^7 normal cells, cells incubated with a hormone-RSA-Sepharose preparation, or normal or unbound cells passed through a histamine-RSA-Sepharose column with or without subsequent incubation with indicated drugs. All incubations for measurement of cyclic AMP were carried out in the presence of theophylline (1×10^{-2} M) in order to block phosphodiesterase. At the end of the incubation, cells were boiled for 10 min, frozen, thawed, and then centrifuged at 3,000 *g* for 15 min. The supernate was separated from the precipitate and cyclic AMP was assayed in appropriate aliquots by a competitive binding assay adapted from that of Gilman (29). All values reported are the means of duplicate or triplicate drug incubations, differing by not more than 8%.

Materials. Prostaglandins were obtained from Alza Corporation, Palo Alto, Calif. All other drugs were obtained commercially (11).

RESULTS

Subtraction of plaque-forming cells by Sepharose preparations. In separate experiments using spleens from BALB/BL mice, cells excluded from columns of histamine-RSA-Sepharose produce between 56 and 84% fewer 19S or 7S plaques than control cells not put over columns (Table I). The high cell to Sepharose ratio and the considerable variability in the effects of different batches of histamine-RSA-Sepharose in part account for the lower mean effect of the columns represented in Table II. In all three experiments tried, the histamine-RSA-Sepharose did not subtract rosette-forming cells from those spleen preparations from which PFC were bound. That the attraction of the insolubilized hormones for the PFC was specific for the hormones and not the carriers is illustrated by: (*a*) the fact that PFC from the same animal attached to the histamine column but were not bound by RSA-Sepharose (Table I), and (*b*) in 10 experiments with each of the Sepharose conjugates represented in Table II, only the histamine, epinephrine, isoproterenol, and PGE_2 preparations significantly altered the plaque-forming population of plated cells. None of the control column preparations (Sepharose, RSA-Sepharose, or activated Sepharose) selectively subtracted substantial numbers of PFC; neither did Sepharose preparations made with norepinephrine and $PGF_{2\alpha}$ alter the plaque-forming ability of excluded cells. There was no significant difference between a substance's ability to subtract direct or indirect PFC from the mixed population; if a Sepharose preparation retained 19S PFC, it also removed 7S plaque-formers. None of the Sepharose preparations used in this study attracted the population of cells capable of forming rosettes.

TABLE I

Simultaneous Determination of the Effects of Two Sepharose Preparations on 19S and 7S Plaques and Rosettes

Column composition (experiment no.)	Percent decrease* of plaques		
	19S	7S	Rosettes
RSA-Sepharose			
1	22	18	4
2	19	18	+3
3	12	8	0
Histamine-RSA-Sepharose			
1	75	84	2
2	56	67	0
3	62	58	0

Experiment number indicates same experiment on same day done 9 days after immunization, e.g. exp. 1 was done on same day with pooled cells from two spleens. Cell experiments used BALB/BL spleen cells.

* Each point represents the average of two separate determinations done in two cell concentrations in triplicate plates (plaques) or tubes (rosettes).

TABLE II

Effect of Hormone-RSA-Sepharose Columns on Plaque Formation

Column material	Decrease of Plaques*	
	19S	7S
	%	
Sepharose	3±1	2±6
Activated Sepharose	5±3	6±2
RSA-Sepharose	14±12	2.7±5
Histamine-RSA-S	54±12	64±19
NE(GA)-RSA-S	32±7	50±23
Isoproterenol-RSA-S	43±21	29±17
NE-RSA-S	7±2	6±4
PG E_2-RSA-S	44±17	28±5
PG $F_{2\alpha}$-RSA-S	4±8	7±10

* Each data point represents the mean±SD of 10 separate experiments done 6 days (for 19S) and 13 days (for 7S) after immunization. In each experiment duplicate points were averaged as explained in Table I. BALB/Bl animals were used. Decrease in plaques per cell from cells excluded from the histamine, NE(GA)-R-S, isoproterenol, and PG E_2 preparations was significantly different from any control ($P < 0.001$ by Student t test). There was no significant subtraction of PFC by NE-RSA-S and $PGF_{2\alpha}$-RSA-S.

The percent of total cells retained by various columns were: Sepharose, activated Sepharose, or RSA-Sepharose, 30–51%; histamine, NE(GA), isoproterenol, 40–60%; norepinephrine and $PGF_{2\alpha}$, 43–55%; PGE_2-RSA-S, 62%.

The percentage of total spleen cells trapped by the various bead preparations were not markedly different, although there was a tendency for insolubilized hormone preparations to bind more cells than Sepharose or Sepharose-RSA alone (Table II). These results indicate that all of the bead preparations nonspecifically trapped about 50% of the cells by an unknown mechanism (perhaps simple mechanical trapping of cells). Since the PFC, which were retained by preparations of insolubilized active hormones, constitute less than 1% of the total number of spleen cells, it is not surprising that their retention does not show up in the percentage figures for total cells.

We also performed preliminary experiments with sequential columns of RSA-S and histamine-RSA-S, which suggested that the second pass of cells over a hormone-bead column did not attract more PFC. Apparently, most of the PFC that will bind to histamine-RSA-S do so in the first pass. We have not attempted to elute PFC from hormone-bead columns with free

TABLE III

Effect of Drug Conjugate-Sepharose Column on 19S Plaque Formation of Three Strains of Mice

Column material	Percent inhibition of plaques*		
	C57BL	BALB/BL	BALB/c
R-S	8±14	14±12	5±6
H-R-S	0±7	54±12	45±13
N(GA)-R-S	2±9	32±7	31±6
PGE_2-R-S	6±3	44±17	58±3

* Each data point represents the mean±SD of 10 experiments in BALB/BL and 5 experiments in each of C57BL and BALB/c mouse spleen cells. Assays were done 6 days after immunization. C57BL were significantly less affected than the other two strains tested ($P < 0.01$, Student t test). Data for each experiment were handled as described in the legend to Table I.

hormones, by analogy with our previous experiments with human leukocytes (10, 11), because such high concentrations of free hormone were required (up to 1×10^{-2} M). The high concentration, presumably necessary because the bead is a polyvalent attractant for cells, would have made any subsequent measures of plaque formation suspect, even with washed cells.

In order to determine whether the Sepharose preparations were able to subtract PFC equally in various strains of mice, spleen cells from the two parental strains of BALB/BL were studied (Table III). Rosette-forming cells were not removed by filtration over histamine-RSA-Sepharose in any of the three strains. The pattern of PFC retention by histamine-RSA-Separose was similar in the BALB/c and BALB/BL strains. In contrast, spleen cells from C57BL/6 mice were not bound by any of the Sepharose preparations.

The following characteristics of the hormone-Sepharose preparations were noted: The percentage of decrease of PFC is a function of the ratio of cells to the amount of histamine-RSA-S (Fig. 1); as the cell number increased, the inhibition decreased. The columns were maximally effective when 10^7 cells were incubated with 0.6 ml of the Sepharose preparation, the ratio used to obtain the results in Table I. However, for convenience, i.e., a reasonable cell yield, in all subsequent experiments, 24×10^6 cells were incubated with 0.6 ml of the Sepharose preparations. There was little effect of any ratio of cells to amount of RSA-S.

Pharmacologic effects of Sepharose preparations. Because stimulation of cells by histamine, beta-catecholamines, and E-series prostaglandins could diminish the ability of cells to form plaques (22), it became important to determine whether the insolubilized amines had pharmacologic properties that could stimulate accumulation of cyclic AMP in leukocytes. Sepharose preparations of RSA linked to either histamine, isoproterenol, norepinephrine, or PGE_1, E_2, or $F_{2\alpha}$ did not cause accumulation of cyclic AMP in BALB/BL cell incubates. Neither did norepinephrine conjugated to RSA via glutaraldehyde have stimulatory properties.

PGE_2-P-S and NE(GA)-P-S stimulated cyclic AMP accumulation in BALB/BL spleen cells. The PGE_2-P-S increased the cyclic AMP content of cells from a mean of 3.7 pmol/10^7 cells (control: polymer—Sepharose incubate) to 9.0 pmol/10^7 cells. Four of seven batches of NE(GA)-P-S significantly ($P < 0.01$) increased cellular cyclic AMP content (Table IV).

We do not yet know why some but not all batches of the NE(GA)-P-S will stimulate cells. Of four separate batches made with the same materials on the same day, only two stimulated the cells. The effect of any one batch of NE(GA)-P-S remained constant; once active, it remained active over weeks of storage, and if inactive, it remained so for as long as the supply lasted (data not shown). The stimulatory effects of the NE(GA)-P-S were partially blocked by phentolamine and totally in-

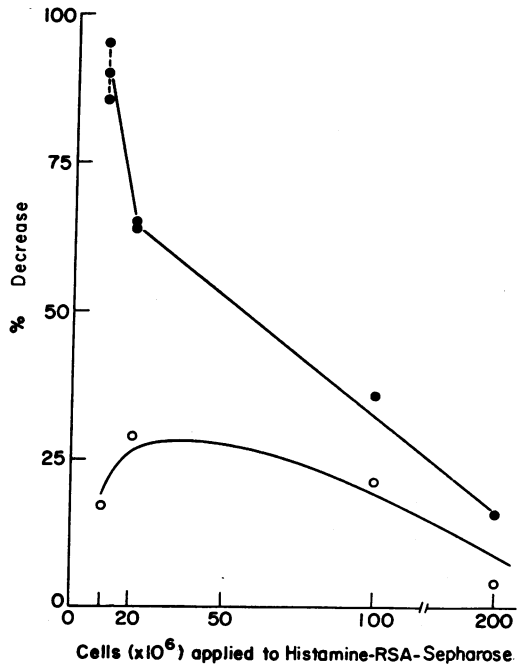

FIGURE 1 Column characteristics relating the ratio of cells ($\times 10^6$)/0.6 ml of histamine-RSA-Sepharose. Equal numbers of unbound cells were plated on agarose, and 19S plaques assessed. Each point represents the mean of triplicate samples. Each sample was plated in triplicate in two cell concentrations. Points compare cells passed through histamine-RSA-Sepharose (●——●) or through RSA-Sepharose (○——○) to control cells not passed through any columns. BALB/BL mouse spleen cells were used 6 days after immunization with SRC.

TABLE IV

Stimulation of Cyclic AMP Accumulation in Leukocytes by NE(GA)-P-S Preparations

Drug	Experiment batch number				
	1	2	3	4	5
Control (cells alone)	4.0±0.1	8.6±0.8	6.3±0.2	7.0±0.4	3.6±0.3
Control (P-S)	2.4±0.02	2.4±0.03	5.9±0.01	2.6±0.01	3.6±0.3
NE(GA)-P-S 1	13.9±0.4				
2		20.6±1.3			
3			2.8±0.4		
4				19.5±0.9	11.5±0.5
5				16±0.6	
6				10±0.6	
7				9.6±0.4	
Supernatant of stored NE(GA)-P-S					3.2±0.1
Supernate of NE(GA)-P-S + cells					3.0±0.4
NE(GA)-P-S + propranolol 10^{-4} M					4.7±0.4
NE(GA)-P-S + phentolamine 10^{-4} M					8.7±0.2
Epinephrine 10^{-4} M					49±1.1
+ propranolol 10^{-4} M					6.6±0.7
+ phentolamine 10^{-4} M					48.5±0.5

* Means±SE of three determinations. Each incubate contained 50 × 10^6 cells and 0.6 ml (25% wt/vol) Sepharose preparation where indicated. All final volumes were 1 ml.

hibited by propranolol (Table IV). Neither the supernate from the stored Sepharose preparation nor that from the incubates of NE(GA)-P-S with cells altered cyclic AMP content of fresh cells incubated with them. Furthermore, when cells were incubated with either [^{14}C]-epinephrine RSA or polymer conjugated to Sepharose, no detectable label was released by the action of the cells on the Sepharose preparations. We conclude from these data that the drug-RSA-Sepharose preparations used in the studies on subtraction of PFC from suspensions of spleen cells pharmacologically did not detectably stimulate the cells, by the criterion of accumulation of intracellular cyclic AMP. It remains possible, however, that increased amounts of cyclic AMP may have been synthesized and released into the extracellular medium.

Are the cells excluded from a hormone-RSA-Sepharose preparation capable of pharmacologic response to free amines? We attempted to determine whether the

TABLE V

Cyclic AMP Accumulation by Mouse (BALB/BL) Spleen Cells

Group	Drug	Experiment number				Mean±SE
		1	2	3	4	
		pmol/10^7 cells				
Mixed spleen cells	None	7.9	3.0	7.4	2.7	5.25±1.39
	Histamine 10^{-4} M	13	4.5	12.5	9.5	9.87±1.95
	PGE_2 10^{-5} M	23	24	27	21	23.75±1.25
Cells excluded from histamine-RSA-Sepharose	None	1.8	0.8	2.3	1.3	1.55±0.32*
	Histamine 10^{-4} M	3	2	3.3	4	3.07±0.41*
	PGE_2 10^{-5} M	8	8	6.2	9.5	7.92±0.67*
Cells excluded from RSA-Sepharose	None	5.8	2		2	3.26±1.26‡
	Histamine 10^{-4} M	11.2	6		6.3	7.83±1.68‡
	PGE_2 10^{-5} M	16.5	31		18	21.8±4.6‡

Each data point represents the average of duplicate samples. The coefficient of variability between duplicates is 8%.

* Significantly different ($P < 0.05$) by paired t test from activity of mixed spleen cells treated with the same drug.

‡ Not significantly different ($P < 0.1$) from activity of mixed spleen cells.

histamine-RSA-Sepharose columns could bind a sufficient number of cells by their receptors for histamine to diminish the response of excluded cells to free histamine or PGE_2. In nine experiments using four separate batches of histamine RSA-Sepharose, we demonstrated that when a cell ratio of 10^7/0.6 ml Sepharose was used, separation of the majority of cells with the histamine receptor could sometimes be accomplished. In four experiments using two batches of histamine-RSA-Sepharose, cells excluded from the histamine-RSA-Sepharose had significantly lower base line concentrations and final concentrations ($P < 0.05$) of cyclic AMP than controls after stimulation by either histamine or PGE_2 (Table V). Neither base line levels nor concentrations resulting from subsequent stimulation of the cells by the same drugs were affected by passing them through RSA-Sepharose columns. In the remaining five experiments using the other two batches of histamine-RSA-Sepharose, the response of excluded cells to histamine or PGE_2 was not significantly altered. Despite the relatively inefficient performance of the later two batches of histamine-RSA-Sepharose, they were as effective in removing PFC as the two batches of Sepharose represented in Table V.

DISCUSSION

We have previously demonstrated that the binding of leukocytes to the histamine and NE(GA)-RSA-Sepharose preparations is likely to be dependent primarily on the hormone and seems unrelated to the carrier protein (10, 11); the present studies confirm this. They also indicate that at least some cells with receptors to histamine and beta-catecholamines produce antibody. By analogy, it is likely that the PGE_2-RSA-Sepharose also binds antibody-forming cells through their receptors, but no competitive pharmacologic antagonists of the binding are available to attempt elution of bound cells from the column. Therefore, a test of the assumption that there are cell surface receptors to prostaglandins cannot yet be made.

Not all of the PFCs were subtracted by any one Sepharose preparation. There are two major possibilities and a number of technical considerations that may explain this phenomenon: first, a number of the antibody-producing cells may not have receptors to any single hormone tested, or second, a substantial number of cells may have receptors to each hormone but technical aspects of the batch chromatography procedure may interfere with binding of all cells with receptors. Perhaps analogies can be drawn with human leukocytes, where the majority of cells that bind to insolubilized hormones bind to both histamine and epinephrine and only a small number are likely to bind to only one insolubilized hormone (11). The data in the present studies may indicate that some mouse spleen leukocytes have receptors for several biologically active substances. A number of insolubilized hormones attracted PFC, and each substance that bound antibody-forming cells did so with about equal effectiveness. It will be of interest to determine whether the majority of PFC can be removed from the mouse spleen cells by passing those that do not adhere to one insolubilized hormone over a series of columns with other hormones on them.

Additional explanations should be considered: that the average depletion of PFC cells by the columns was only about 50% of the total is probably due in part to the experimental conditions. To obtain an adequate yield of cells from the column within a reasonable time, we had to work at ratios of cells to Sepharose beads that produced submaximal depleting effects (Fig. 1). With most preparations of histamine-RSA-Sepharose, the cells that were not bound to the columns even under optimal conditions could still respond to histamine and prostaglandin stimuli. Certainly in most experiments not all cells with receptors were removed from the cell mixture. These effects probably indicate that various preparations of hormone-RSA-Sepharose have sufficiently variable molar ratios of amine to RSA to alter their effective affinity for cells that can be attached to the polyvalent attractant (11), and that morphologically similar cells with specific receptors for a given amine have a spectrum of affinities for the same attractant. In the latter instance, a substantial number of the cells with plaque-forming ability seem to have a high enough affinity for the histamine attractant to be preferentially and selectively removed from the mixture without lowering the total number of cells responsive to either histamine or prostaglandin. In the instances when response to histamine was lowered, so was the response to prostaglandin. Such findings may be consistent with the data in human leukocytes that indicate that receptors for a number of biogenic amines and the prostaglandins may be present on or in the same cell (11). The data are also consistent with the findings that a number of free hormones prevent plaque formation (22), and the same hormones can be insolubilized and used to attract PFCs.

The insolubilized hormones attract 7S and 19S PFC equally but the free hormones do not appear to inhibit 7S plaque formation as well as they inhibit 19S (22). The apparent discrepancy may be largely artifactual. The plaque assay produced considerable variability in these experiments and therefore can be perceived only as a qualitative indicator of drug or column effects. No attempt has been made to compare either the results produced by free versus the binding produced by conjugated and insolubilized hormones, or the inhibition of 19S versus 7S plaque formation. These studies await more precise measurement of total antibody concentra-

tions per cell. Furthermore one might expect more pronounced and repeatable effects of insolubilized hormones than free hormones, since the affinity of the cell for the hormone seems to increase as the conjugate becomes polyvalent (11). In addition, a cell incubated with a single molecule of a conjugate of RSA-hormone actually is exposed to about 50 molecules of hormone (11). The "real" local concentration of hormone created by a conjugate of the hormone may be many times greater than that created by a similar amount of free hormone per milliliter of suspension.

The data in Table III indicate that strain differences may be associated with alterations in the affinity of cells for the insolubilized hormones. However, despite the inability to subtract PFCs from C57BL mouse spleen cells, the cells did respond to free histamine, epinephrine, and PGE_2 (22). They therefore have receptors for the biogenic substances used here. The increase in cyclic AMP content of the C57BL cells and inhibition of plaque formation during such an increase were similar to both such changes obtained in cells from the related strains of mice (22). The pharmacologic effects of any drug seemed to be equivalent in each strain tested.

The experiments that showed that only some of the PFC capable of responding to free hormone were removed from cell mixtures by histamine-RSA-Sepharose indicate that cells from a given spleen with receptors for the same hormone may have a spectrum of affinity. We cannot determine whether all cells that produce antibody have receptors to the endogenous hormones tested. Neither do we know whether the ability to separate antibody-producing cells on the basis of their hormone receptors is dependent on the antigen used for immunization.

Each hormone-RSA-Sepharose preparation that bound antibody-forming cells was made of hormone agonists for which corresponding leukocytic pharmacologic receptors have been found. Thus histamine, beta-catecholamines (but not alpha-mimetic catecholamines), and the E-series prostaglandins (but not the F-series) increase cellular cyclic AMP (14) and inhibit plaque formation (22). None of the Sepharose preparations used in this study to separate PFC from others was pharmacologically active or noxious to the cells. Therefore, the effects of the columns must have been on the basis of simple physical separation of cells.

Free norepinephrine in high concentration stimulates leukocyte cyclic AMP, yet NE-RSA-Sepharose did not bind antibody-producing cells. Only when norepinephrine was bound through glutaraldehyde to the carrier protein and Sepharose was there attraction of cells with antibody-producing properties. This may be explained on three bases: (*a*) When norepinephrine is attached via ECDI to a carrier protein, the primary amine is probably the functional group bound to the carrier. The coupling process produces an amide bond between the amino group of the hormone and a carboxyl residue of either aspartic acid or glutamic acid of the protein (11). This linking process drastically reduces the basicity of the amino groups and therefore the binding properties of the hormone. (*b*) When the amino group of the hormone is coupled via glutaraldehyde, the basic function of the hormone is preserved since the imino bonds formed by the interaction of the aldehyde with the amine groups are completely reduced to alkylamines by sodium borohydride. (*c*) When the hormone is bound via glutaraldehyde to a flexible copolymer, the chemical configuration (11) and the pharmacologic properties (Table IV) more closely resemble epinephrine than norepinephrine. Since epinephrine is a more potent stimulator than norepinephrine of both human and mouse spleen leukocytic adenyl cyclase, the binding of cells by NE(GA)-RSA-Sepharose but not by NE-RSA-Sepharose might be understandable.

Some evidence suggests that receptors to histamine do not develop on or are not exposed on the precursors of antibody-forming cells. Spleen cells from either primed or unprimed donor mice have been passed over a histamine-RSA-Sepharose column and the unbound cells transferred to irradiated, syngeneic recipients that are simultaneously immunized with SRC. Surprisingly, these transferred cells ultimately produce more than three to four times as many recipient PFC responses as do transfers of equal numbers of control cells or cells excluded from an RSA-Sepharose column (23). If the precursor cells to antibody formation had receptors to the amine, we would have expected to retain them on columns of the conjugated hormones. Thus the transfers done with cells excluded from histamine-RSA-Sepharose should have resulted in fewer PFC than controls. Because the enhancement seen in these experiments cannot be accounted for on the basis of cell concentration, we tentatively concluded that receptors for the amines may appear some time after the cell has been committed to production of antibody, i.e., after immunization.

Some mention of the data on rosettes is warranted. One would have expected some binding effect of the hormone-RSA-Sepharose on rosette formers if rosette formers express receptors to SRC antigen, and if some portion of these also are antigen-sensitive cells (30). There was no effect of the columns on rosette formers. The observation that hormone-Sepharose columns retained PFC but not rosette formers supports other published results whcih suggested that PFC and rosette formers represent distinct populations of immunocompetent cells (25, 30, 31).

Although relatively little attention has been focused on the effects of small hormones on the immunologic

process, it is not surprising that cells involved in the process possess receptors to histamine, beta-catecholamines, and PGE_2. These agonists also modulate the cytolytic effects of lymphocytes sensitized on fibroblasts or mast cells, inhibit IgE-mediated release of histamine from mast cells, and prèvent antibody release from the same cells studied in this report. Our studies indicate that when cells are separated on the basis of their physiologic receptors to these small hormones, a population of cell that can control an immunologic response in vivo (23) and produce antibody is removed from the general group (present study). How mediators of an inflammatory response modulate an immunologic process is presently being explored. Whether such modulation has any importance in vivo remains to be seen.

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