REQUIREMENT FOR VASOACTIVE AMINES FOR PRODUCTION OF DELAYED-TYPE HYPERSENSITIVITY SKIN REACTIONS*

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Injection of antigen into the dermis of the flank of an appropriately immunized rat, guinea pig, monkey, or man results, 24–48 h later, in the formation of an erythematous, indurated lesion. Similar skin testing of immunized mice generally fails to produce such lesions (1–3). The explanation for this particular difference between mice and men is unknown but there is reason to believe that it may not stem from differences in immunologically competent cells. Two observations support this view. (*a*) Appropriately immunized mice exhibit antigen-specific delayed-type hypersensitivity (DTH)[1] reactions when the site of elicitation is the foot pad (4) or the ear (5). (*b*) Mice exhibit most other manifestations of cell-mediated immunity, in a normal fashion, despite their failure to produce DTH reactions in the flank skin. Thus, mice must have appropriately reactive T cells but there may be some difficulty in delivering the cells required for the production of DTH reactions to the flank skin. In support of this notion, it has been shown that if peritoneal exudate cells are added to the eliciting dose of antigen placed in the flank skin the lesions that result are morphologically indistinguishable from those of classical DTH (2). Appropriate immunization and the inclusion of the specific antigen with the exudate cells are required for the production of these lesions. It thus appears that sensitized T cells may react with antigen in the flank skin of mice but that the subsequent migration of nonimmune cells, which is required for production of the lesions (6–11), does not normally ensue.

It has recently been shown that basophils comprise a significant portion of the inflammatory exudate in almost all types of DTH reactions of guinea pigs and man (12) and can play a functional role in these reactions (13). Since basophils are important carriers of vasoactive amines, we considered it possible that the importation of these substances to the site of delayed reactions might play an important role in the development of the lesions. If this were true, it could explain why mice usually fail to exhibit delayed lesions in their flank skin, as this species is markedly deficient in circulating basophils (12, 14). Production of

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[1] *Abbreviations used in this paper:* DNFB, dinitrofluorobenzene; DTH, delayed-type hypersensitivity; 5-HT, serotonin (5-hydoxytryptamine); MAO, monoamine oxidase; PCA, passive cutaneous anaphylaxis.

lesions in the foot pads and ears might result if these areas were particularly rich in mast cells which could substitute, at least in part, for recruited basophils.

Therefore, we have examined the role of vasoactive amines in the production of DTH reactions in the foot pads of mice. Our results show that the skin of the mouse's foot pad contains many more serotonin (5-hydroxytryptamine)-containing mast cells, per unit weight, than does the flank skin and that pretreatment of mice, with the monoamine depleting drug reserpine, blocks the elicitation of DTH. This effect of reserpine can itself be blocked by monoamine oxidase (MAO) inhibitors, confirming that the action of reserpine depends upon the intracellular release and catabolism of monoamines by MAO. It is known that the monoamine serotonin is the principal mediator of anaphylactic reactions in the mouse (15, 16). We suggest that this substance may also play an important role in the production of the lesions of DTH, perhaps by acting on the endothelial cells of the postcapillary venules (17) and permitting the egress of macrophages or their precursors from the blood into the sites of delayed inflammation. Macrophages which ordinarily do not pass through postcapillary endothelium, and thus do not recirculate, are the cells principally responsible for the swelling and induration of DTH reactions (6–11).

Materials and Methods

Mice. Mice were 6- to 8-wk old male BDF_1 (C57BL/6 × DBA/2) from the Jackson Laboratories, Bar Harbor, Maine. They were rested 1 wk in our animal facilities before use.

Immunizations

SHEEP RED BLOOD CELLS (SRBC). Groups of five–six mice were immunized under light ether anesthesia by intravenous injection of 0.2 ml of a freshly washed suspension of SRBC (0.01%; about 2×10^5 cells) in saline, or by separate subcutaneous 0.05-ml injections into the four proximal limbs of an emulsion containing (in equal parts) 10% SRBC and CFA (H37Ra, Difco Laboratories, Detroit, Mich.), fortified with 3 mg/ml ground mycobacterium tuberculosis.

CONTACT REACTIONS. A single application of 0.1 ml of 3% oxazolone (2-phenyl-4-ethoxymethylene oxazolone, Gallard, Schlesinger Corp., Carle Place, N.Y.) in ethanol was made to the freshly shaved abdominal skin of unanesthetized mice (5) or 0.02 ml of 0.5% dinitrofluorobenzene ZDNFB, Eastman Chemicals, Rochester, N.Y.) in a 1:4 mixture of acetone and olive oil was made on 2 consecutive days (18).

Foot Pad and Ear Skin Testing

FOOTPAD. Mice immunized intravenously with 0.01% SRBC were tested 4 days later [this has been shown to be the time of optimal delayed foot pad responses produced by this mode of immunization (19)]. Separate groups of mice immunized with SRBC in CFA were tested at day 4 and day 10. Freshly washed 1-wk old SRBC (20% in saline) were injected (0.03 ml) into the ventral foot pad after foot pad thickness had been measured using a micrometer (Brown & Sharpe Mfg. Co., No. Kingston, R. I.). Subsequent 24-h foot pad thickness was compared to the original measurement and the percent change calculated.

EAR SKIN. Contact reactions of the ear skin were elicited by applying to each side of both ears a drop of freshly prepared 3% oxazolone in olive oil 7 days after immunization (5) or 0.2% DNFB in acetone-olive oil 5 days after initial immunization (18). Micrometer-measured ear thickness at 24 hs was compared to ear thickness observed before contact testing, and percent change calculated. Student's t test was used to compute statistical significance ($P < 0.05$).

Reactions of Passive Cutaneous Anaphylaxis (PCA) (20). Mice were contact-sensitized with oxazolone and boosted with similar additional applications to the abdominal skin at days 7 and 14. Serum harvested at day 21 was diluted twofold in saline starting at 1:20. 0.02 ml of four separate dilutions were injected intradermally into the shaved skin of groups of three to four Swiss mice anesthetized with ether. 2 h later 0.2 ml saline containing Evans Blue Dye (1/4%) and 0.5 mg

oxazolone-human serum albumin (21) was injected intravenously. 30 min later mice were killed and the skin reflected to read the diameter and intensity of extravasated dye.

Drugs

RESERPINE (5 MG/KG). The amine-depleting drug reserpine (22, 23) (Serpasil for parenteral injection, Ciba, Summit, N.J.) was injected as a single dose 6–12 h before testing of the skin or foot pad. Reserpine has a number of advantages as an experimental tool with which to manipulate monoamines. It has a long duration of action on amine storage, one which outlasts the presence of the drug in the animal. This permits possible direct effects of reserpine to be differentiated from the effects secondary to amine depletions. Reversal or antagonism of reserpine's action by MAO inhibitors permits a second check on drug specificity. Moreover, the well-characterized depletion of amines by reserpine is intrinsically a more specific approach than would be, for example, the use of antagonists which may have several actions, not all of which are known, and which may block some, but not all of the amine receptors; multiple 5-HT receptors have recently been recognized (24, 25).

MAO INHIBITORS. *Pheniprazine* (10 mg/kg) (Lakeside Laboratories Milwaukee, Wis.) was injected intraperitoneally 30–60 min before injection of radiolabeled 5-HT (see below). *Pargyline HCl* (100 mg/kg) (Abbot Laboratories, No. Chicago, Ill.) was dissolved in saline and administered intraperitoneally 30–60 min before injection of radiolabeled 5-hydroxytryptamine (5-HT) (see below). *Nialamide* (100 mg/kg) (Sigma Chemical Co., St. Louis, Mo.) was freshly solublized in 3 M HCL and the pH was adjusted to 5.0 by the addition of 1 N NaOH. Water was added to bring the concentration to 10 mg/ml and the diluted drug was administered a few minutes before reserpine and every 6 h thereafter to block the MAO catabolism of released monoamines.

BURIMAMIDE. Burimamide (500 μmol/kg), a blocker of Histamine-2 (H_2) receptors (26) (gift from Smith Kline & French Labs, Welwyn Garden City, Herts, England) was administered subcutaneously 1 day before foot pad testing.

6-HYDROXYDOPAMINE HYDROBROMIDE. 6-Hydroxydopamine (100 mg/kg) (Sigma Chemical Co., St. Louis, Mo.), which produces a chemical sympathectomy (27), was dissolved in saline containing ascorbic acid (1 mg/ml) and injected subcutaneously twice, 48 and 24 h before testing the treated mice.

Determination of the Uptake of 5-HT

SCINTILLATION COUNTING. (*a*) *In the skin:* Six mice were pretreated at 48 and 24 h with 6-hydroxydopamine and with the MAO inhibitor pheniprazine. 30 min after the injection of pheniprazine the animals received 0.1 mCi of tritiated 5-HT ([^{3}H]5-HT in 0.1 ml saline; serotonin binoxalate, New England Nuclear Corp. Boston, Mass., 1.33 Ci/mmol) intravenously. 2 h later the mice were killed by cervical dislocation and skin was removed from the feet, flanks, and ears. Care was taken to avoid including cartilage with the samples of ear skin. Tissues were quickly weighed and homogenized in 5.0 ml of 70% ethanol. The resulting suspensions of tissue were allowed to stand overnight at 4°C. After centrifugation at 12,000 *g* for 20 min, aliquots were removed for chromatographic analysis and liquid scintillation counting. This procedure has been shown to extract better than 95% of the radioactive 5-HT from tissues (28). Thin-layer chromatography done with butanol:acetic acid:water (12:3:5) or isopropanol:ammonia:water (10:1:1) on cellulose (Eastman Chemicals) revealed that radioactivity measured in the ethanolic extracts was taken as an assay of the amount of [^{3}H]5-HT in the tissue and was expressed as dpm/g. Samples were dissolved in 15 ml of "Aquasol" (New England Nuclear Corp.) for liquid scintillation counting and corrected for quench by external standardization.

(*b*) *In tumors:* Mice carrying a mast cell tumor (P-815) were injected intraperitoneally with 100 mg/kg of pargyline to inhibit MAO. 1 h later each mouse was injected intraperitoneally with 0.1 mCi of [^{3}H]5-HT. Another two mice carrying the mast cell tumor were injected with 5 mg/kg of reserpine 4 h before injection of [^{3}H]5-HT. One of these animals was given pargyline as above and the other received no other drugs before injection of the isotope. 2 h after the injection of [^{3}H]5-HT all mice were killed, their abdomens opened and washed with 2.0 ml of iced Krebs solution, and the tumor cells collected.

After collection in Krebs solution, the cells were pelleted by centrifugation at 1,200 *g* for 10 min in a refrigerated centrifuge. The supernate was discarded, the cells were drained, and the pellets were weighed. 5 ml of 70% ethanol was then added to the cells. The cells were homogenized,

allowed to stand overnight, centrifuged again as described above, and aliquots of the supernate were taken for liquid scintillation counting and chromatographic analysis.

RADIOAUTOGRAPHY. Experiments were designed to determine if the assumption that all of the tritiated 5-HT in the skin was localized to mast cells was valid. This was done by radioautography. Methods were the same as those described by Gershon and Ross (29, 30).

Five mice were injected intravenously with 5.0 mCi of tritiated 5-HT creatinine sulfate (Amersham/Searle Corp., Arlington Heights, Ill.; spec act 5–18 Ci/mmol). Injection volume was 0.1 ml. Pheniprazine was given to inhibit MAO 60 min before injection of the isotope. Segments of skin were removed after 2 h and fixed in iced 6.25% gluteraldehyde made hypertonic by the addition of 9% sucrose and buffered to pH 7.4 by 0.1 M phosphate buffer. Under these conditions of fixation, movement of [^{3}H]5-HT is abruptly halted (27, 29). [^{3}H]5-HT is preserved in situ but its metabolites are not. After fixation for 2 h in gluteraldehyde, tissues were washed briefly in the sucrose-containing buffer solution and were postfixed for 1 h in 1% OsO_4. The OsO_4 was buffered to pH 7.4 with 0.1 M phosphate buffer containing 9% sucrose. The tissues were then rapidly dehydrated and embedded in epoxy resin (Epon 812). Sections were cut at 0.5 μm, placed on glass slides, and coated with Ilford L4 emulsion (diluted 1:1) by dipping. The emulsion-coated slides were exposed for 1 wk in a dry, light-tight box under an atmosphere of 100% CO_2. After photographic development and processing, slides were stained with toluidine blue for light microscopic examination. None of the processing of the fixed tissues extracts significant quantities of radioactive material (29–31).

Histochemical Demonstration of 5-HT. Tissue was examined histochemically for the presence of 5-HT by the formaldehyde-induced fluorescence technique of Falck and Hillarp (see 32 for refs.). Treatment of the frozen-dried tissues with paraformaldehyde was modified according to the procedure of Fuxe and Jonsson (33). Briefly, small pieces of tissues were quenched in melting Freon 22 that had previously been cooled with liquid nitrogen. Tissues were freeze-dried overnight in a Pearse-Edwards freeze drier, and then exposed at 80°C to formaldehyde gas generated first from paraformaldehyde equilibrated at 70% and then at 90% relative humidity. Tissues were embedded in hydroxybutyl methacrylate, sectioned at 3 μm, and mounted on slides in nonfluorescent immersion oil under quartz cover slips for fluorescence microscopy. The microscope was fitted with a vertical illuminator (Leitz Ploem system, E. Leitz, Inc., Rockleigh, N.J.). Identification of the fluorophore as that of 5-HT was done by microspectrofluorophotometry (Leitz-Schoeffel microspectrofluorophotometer). Activation maxima were at 385 and 420 nm and the emission maximum was at 520 nm. Controls included tissues heated as above at 80°C but not exposed to paraformaldehyde. Hairs fluoresced in the absence of formaldehyde treatment and was therefore due to autofluorescence and not to the presence of a monoamine.

Histochemical Demonstration of Histamine. Tissue also was examined histochemically for the presence of histamine by the *o*-phalaldehyde-induced fluorescence technique of Ehinger and Thunberg (34) as modified by Cross et al. (35). Freeze-dried tissue was embedded in paraffin, sectioned at 5 μm, deparaffinized with xylene, exposed to *o*-phalaldehyde vapor at 100°C, and subsequently humidified. Sections were mounted in Entellan (Merck Chemical Div., Merck and Co., Inc., Rahway, N.J.) and examined by fluorescent microscopy using a vertical illumination and a TK 400 dichroic beam splitting mirror and a K 430 suppression filter.

Results

Distribution of Mast Cells at Various Skin Sites in the Mouse. We studied mast cell distribution by several techniques: (*a*) After chemical sympathectomy for the prevention of [^{3}H]5-HT uptake by adrenergic nerves, we measured the uptake of [^{3}H]5-HT in various regions of the skin. The results (Table I) indicate that the concentration of mast cells, as determined by their ability to take up radioactive 5-HT, is in the order of foot greater than ear greater than flank.

The quantitative results obtained by scintillation counting were checked by radioautography to determine where in the skin the 5-HT had localized. More [^{3}H]5-HT was given (50 times as much) to be certain that small amounts bound in unexpected locations would not be overlooked. The only labeled structures

TABLE I

Localization of Injected [^3H]5-HT at Various Skin Sites

Region of skin	dpm/g ± SE
Foot	173,000 ± 42,000
Flank	15,400 ± 1,500
Ear	47,200 ± 14,000

P values: ear vs. foot = <0.02; foot vs. flank = <0.01; ear vs. flank = <0.05.

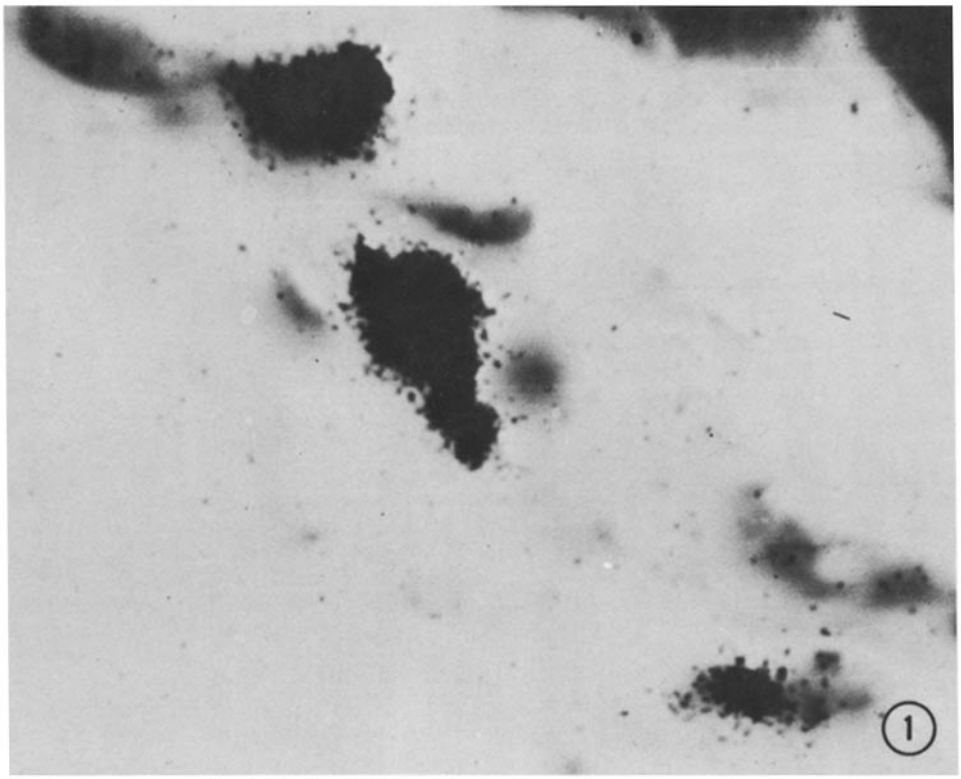


FIG. 1. Radioautograph of the subcutaneous tissue of a mouse which was injected with radioactive serotonin ([^{3}H]5-HT). Only certain cells localized [^{3}H]5-HT and were heavily labeled, while nearby cells took up no label. Staining with toluidine blue showed that the cytoplasm of the heavily labeled cells contained numerous large metachromatatic granules. Variation of the plane of focus at lightly labeled areas of these cells revealed that the grain pattern corresponded to the shape and position of the granules (× 950).

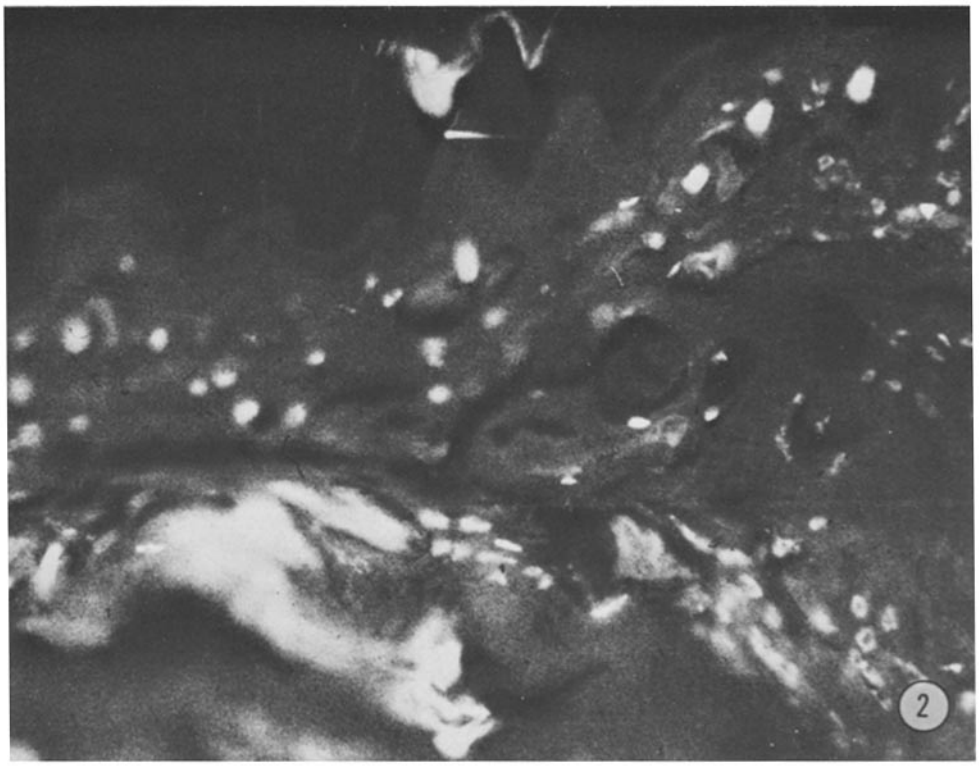
that we could find in any of the slides of skin were mast cells (Fig. 1). These cells could be readily identified by their metachromasia in the toluidine blue-stained sections under the layer of photographic emulsion. The cells were very heavily labeled. No evidence of uptake of 5-HT by perivascular sympathetic nerves could be found. If animals were treated with reserpine (5 mg/kg) 1 day before injection of [^{3}H]5-HT, no labeled mast cells were found. These observations support the

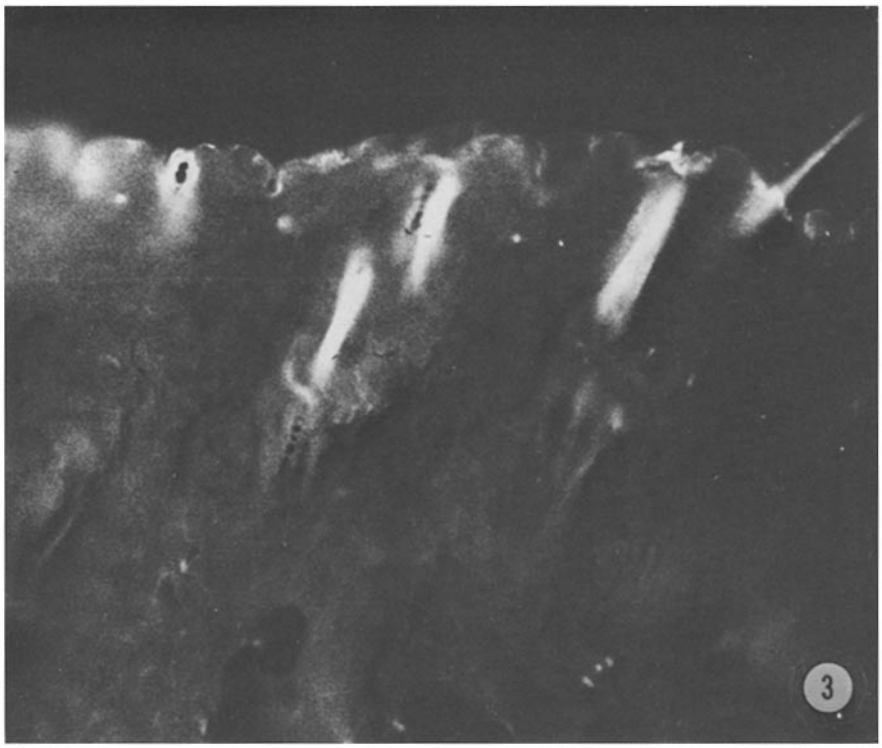
view that mast cells account for the uptake of labeled 5-HT by the skin. Therefore, the amount of tritiated 5-HT found in the skin probably is a function of the number of activity of the mast cells in the area.

(*b*) Independent confirmation of the quantitative data derived from studies of the uptake of [^{3}H]5-HT, suggesting that mast cells were most numerous in the skin of the foot, was sought. Mast cells of mice and rats are known to contain 5-HT and histamine (see 14). Therefore, 5-HT and histamine was demonstrated in sections of the skin histochemically in order to visualize mast cells and to estimate their numbers. By means of formaldehyde (5-HT) and *o*-phalaldehyde (histamine)-induced histofluorescence, many brightly fluorescent mast cells could be seen in sections of the skin of the foot, especially on the dorsal surface clustered around autofluorescent hair follicles (Fig. 2). Far fewer fluorescent mast cells were found in sections of flank skin (Fig. 3), while ear skin had intermediate numbers. These results confirm those above, that mast cells in the skin which contain 5-HT are far more numerous in the skin of the feet and somewhat more numerous in the skin of the ears than of the flank, and that histamine-containing mast cells have the same distribution. Mast cells identified by numerous cytoplasmic metachromatic granules in paraffin sections viewed with an ordinary light microscope after Helly's fixation and Giemsa staining (36) were similarly distributed.

The Effect of Reserpine on the Uptake of 5-HT. Radioautographic observations indicated that pretreatment of mice with reserpine totally ablated the capacity of the mast cells to take up and/or store 5-HT. We also studied the ability of reserpine to block the uptake of [^{3}H]5-HT by P815 mastocytoma cells. As a specificity control for the uptake of the 5-HT by the mastocytoma cells, we used a different mouse tumor, sarcoma I. The results of this experiment (Table II) show that the mast cell tumor took up more 5-HT than did sarcoma I cells. Reserpine antagonized the ability of the tumor cells to accumulate 5-HT and this antagonism was partially overcome by the administration of the MAO inhibitor pargyline.

Effect of Reserpine Pretreatment on the Ability of Mice to Make a DTH Response to SRBC. Mice were immunized intravenously with 0.2 ml of a 0.01% suspension of SRBC. Their ability to mount a DTH response was tested 4 days later by injecting 0.03 cc of SRBC into the foot and measuring the increase in foot pad thickness 24 h later. Some of the mice were treated with reserpine alone, some with reserpine plus the MAO inhibitor nialamide, some with 5-hydroxydopamine as a control for the effects of reserpine on norepinephrine (27), and some with burimamide, a blocker of histamine-2 (H_2) receptors (26) [antihistamines which work on H_1 receptors are known not to affect reactions of delayed hypersensitivity (37) and in any case the mouse is extraordinarily resistant to histamine (see 16)]. The results of this experiment (Table III) show that reserpine treatment abrogated the DTH response occurring 24 h after challenge with antigen, that the MAO inhibitor nialamide partially reversed this suppression, and that the depletion of norepinephrine alone and H_2 blocking were without effect. We subsequently found that delaying the injection of an MAO inhibitor until 6 h after administration of reserpine fails to reverse the effect of reserpine on DTH reactions but does reverse the central nervous system effects.





FIGS. 2 and 3. Formaldehyde induced fluorescence of serotonin (5-HT) in the skin of mice. 5-HT-containing cells were quite numerous in the dorsal foot skin (Fig. 2), and were localized to the upper dermis where mast cells were most numerous as judged by light microscopy. The cells at the lower right demonstrate that 5-HT was localized to the cytoplasm (× 100). Fluorescent cells were noted only occasionally in the flank skin (Fig. 3). [(*o*-phalaldehyde-induced fluorescent cells (histamine-containing) were similarly distributed).]

TABLE II

Uptake of [^3H]5-HT by Different Drug Treated or Control Tumor Cells

Cells (and drug treatment)	dpm/g (mean of cell pools)
Sarcoma I (none)	238,000
Mastocytoma cells (none)	1,110,000
Mastocytoma cells (reserpine)	33,000
Mastocytoma cells (pargyline + reserpine)	346,000

TABLE III

Reserpine Inhibition of DTH (0.01% Intravenous SRBC Immunization)

Drug	24 h foot pad thickness
	% increase ± SE
None	57 ± 5
Reserpine	4 ± 2
Reserpine + nialamide	31 ± 6
6-Hydroxy-dopamine	64 ± 6
Burimamide	64 ± 7

TABLE IV

Reserpine Inhibition of DTH (SRBC + CFA Immunization)

Drug	24 h foot pad thickness	
	4 days	10 days
	% increase ± SE	
None	23 ± 1	42 ± 6
Reserpine	7 ± 3	−1 ± 3

We tested the possibility that the low dose SRBC immunization schedule we used was not truly representative of a DTH response. To do this, we immunized mice with SRBC in CFA and tested the ability of reserpine to block the delayed-hypersensitivity response in the footpad 4 and 10 days after immunization. The results (Table IV) show that reserpine could block the delayed response to this form of immunization as well as it could to the low dose immunization.

Effect of Reserpine on Contact Reactions. We also tested the ability of reserpine to inhibit reactions of contact hypersensitivity occurring on the mouse ear. The results (Table V) show that reserpine was also able to inhibit these forms of DTH, although the inhibition was not as complete as in the SRBC system.

Effect of Reserpine on Passive Cutaneous Anaphylaxis. Our results indicate that reserpine can significantly suppress several types of DTH reactions and that MAO inhibition can partially prevent this suppression in at least one form

TABLE V

Reserpine Inhibition of DTH (Contact Hypersensitivity)

Immunization	Reserpine	24 h ear thickness
		% increase ± SE
Oxazolone	−	90 ± 9
Oxazolone	+	38 ± 10
DNFB	−	37 ± 6
DNFB	+	15 ± 5

TABLE VI

Reserpine Inhibition of PCA Reactions

Drug	Diameter (in mm) ± SE and intensity of blueing (0–4+)			
	Antibody dilution	1:20	1:40	1:80
None	13 ± 1 (3+)	10 ± 1.5 (3+)	8 ± 1.5 (2+)	
Reserpine	3 ± 2 (1+)	0	0	
Reserpine + nialamide	0	0	0	

of DTH. This latter point was of particular interest since reserpine is known to act by causing stored amines to leak from the granules of mast cells into the cytosol, where they are inactivated by MAO (38). Since inhibition of MAO after reserpine would lead to the accumulation of free amine in the cytosol of mast cells, this suggested that some form of nongranule release of amines might be occurring. We therefore tested whether reserpine could inhibit PCA reactions which are known to be dependent upon exocytosis of mast cells granules (39) and to see whether nialamide could reverse that form of inhibition. The results of three pooled experiments (Table VI) show that reserpine significantly inhibited PCA reactions, but in contrast to the DTH reactions, nialamide was now unable to reverse this inhibition.

Effect of Adrenalectomy on the Ability of Reserpine to Block Reactions of Delayed Hypersensitivity. Although our results show that reserpine treatment affects the ability of mast cell granules to bind and store 5-HT, we considered the possibility that this was not causatively related to its ability to block reactions of DTH but that there might be an indirect action. We had ruled out effects secondary to reserpines tranquilizing action by the differential protection afforded on behavioral effects and DTH by delayed treatment with MAO inhibitors. Another indirect action could come from stress and the subsequent release of corticosterone from the adrenals. We therefore tested the ability of reserpine to inhibit DTH reactions in adrenalectomized mice. The results of this experiment (Table VII) show that adrenalectomy was without significant effect on the DTH in either untreated or reserpine-treated mice. Therefore, nonspecific stress was not a significant factor in our results.

Effect of Adoptive Transfer of Immune Spleen Cells from Reserpine-Treated

TABLE VII

Effect of Reserpine on DTH in Adrenalectomized Mice (0.01% SRBC Immunization)

Adrenalex	Reserpine	24 h foot pad thickness
		% increase ± SE
−	−	54 ± 7
−	+	−3 ± 1
+	−	62 ± 5
+	+	2 ± 1.5

TABLE VIII

DTH Transfer into and out of Reserpine-Treated Mice (Donors immunized with 0.01% SRBC)

Reserpine treatment	24 h foot pad thickness in recipients
	% increase ± SE
(1) None	44 ± 4
(2) Recipient	1 ± 3
(3) Donor	40 ± 5
(4) "Donor"	5 ± 2 (reaction in donor)

Mice. We performed a more direct test for secondary effects of reserpine treatment by doing adoptive transfer experiments. We immunized mice with SRBC and treated some of them on day 4 with reserpine. 8 h after injection of reserpine, we harvested the spleens and transferred the resultant cell suspension (one spleen: two recipients) to normal nonimmune syngeneic mice, some of which had been pretreated with reserpine at the same time as the donor mice. The results (Table VIII) show that treatment of donors with reserpine abrogated their ability to mount a DTH response (line 4). However, spleen cells of the reserpine-treated donors, who themselves could not make a DTH response, were able to transfer DTH to normal recipients (line 3), as well as spleen cells from donors who had not been treated with reserpine (line 1). If however, the recipients had been treated with reserpine, the transfer of immune spleen cells failed to convey the ability to mount a DTH response to the recipient (line 2). Therefore, reserpine acts on cells of the host, not on the immunocompetent cells of the donor.

Discussion

We would like to set forth some previously made observations and those elucidated in the present study, and from them put forth a hypothesis to explain certain elements of delayed hypersensitivity reactions and their relationship to other immunological phenomena. Pertinent observations made in previous studies include the following: (*a*) mice are relatively deficient, compared to other animals, in their ability to make DTH reactions in the flank skin (1–3); (*b*) they nevertheless make excellent DTH reactions when the eliciting antigen is placed

in the foot pad (3, 4) or on the ear (3, 5); (*c*) mice are relatively deficient in basophils, circulating cells which carry vasoactive amines (12, 14), and these cells are often found in the cellular exudate in DTH reactions of other mammals (12); (*d*) vasoactive amines allow intravascular contents to pass into the tissue by causing the endothelial cells of postcapillary venules to contract and separate (17); (*e*) the predominant recirculating lymphocyte found in the mouse thoracic duct is a T cell (40), indicating that these cells are especially equipped to leave the blood and enter the lymph; (*f*) T cells play an important role in the recruitment and/or entrapment of cells from the circulation to lymphoid tissues draining sites of exogenously administered antigen (41).

Observations made in the present study include the following: (*a*) mast cells which contain vasoactive amines are particularly numerous at skin sites in the mouse where DTH reactions are most easily elicited; (*b*) vasoactive amine depletion by reserpine severely inhibits various DTH reactions; (*c*) the techniques used for depletion of vasoactive amines do not alter the ability of T cells of treated mice to adoptively transfer DTH reactions to normal recipients. Thus, the site of action of vasoactive amine depletion is not at the level of the effector T cell; (*d*) pharmacological treatments which cause depletion of vasoactive amines from mast cells granules, but which favor accumulation of free amine in the cystosol, (reserpine plus MAO inhibitors) eliminate reactions of passive cutaneous anaphylaxis but not those of delayed hypersensitivity.

To account for these observations, we propose that T cells are especially equipped to traverse the endothelium of postcapillary venules, which they continually do. If they are appropriately immune and meet the specific antigen outside of the circulation, they release a factor that causes nearby mast cells and/or basophils to release their vasoactive amines. If basophils are absent, mast cells acquire added importance. The released amines cause the endothelial cells of the postcapillary venule to separate, allowing the rapid egress from the blood of cells which do not normally recirculate. Nonrecirculating cells coming from the bone marrow are the predominant cell type that causes the swelling and induration of a delayed hypersensitivity reaction (6–11). There probably is an additional element of a T-cell-released chemotactic substance or substances which attract the appropriate cells to the site (42). However, the release of these chemotactic substances is insufficient in itself to permit the secondarily arriving cells to enter the reaction site, unless the walls of the postcapillary venules have been acted upon by vasoactive amines.

This T cell-to mast cell-to postcapillary venule reaction is also probably very important in the general regulation of lymphocyte traffic. [We have evidence that depletion of 5-HT from the mouse abrogates the antigen-driven trapping of lymphocytes in the lymph nodes but not in the spleen (Gershon and Kondo, unpublished observations).] This may be why workers studying the mode of entrance of lymphocytes into nonantigen-stimulated lymph nodes find that cells go through, rather than between, endothelial cells (43). Those who study the mode of entrance in lymphoid tissue of the gut where there is constant antigenic stimulation have found that the cells go between the endothelial cells of the postcapillary venule (44). There may be a continuing antigen-to T cell-to mast cell reaction going on in lymphoid tissue draining the gut, which accounts for the different mode of circulation.

Although our results do not bear directly on the mechanism by which T cells cause mast cells to release their vasoactive amines, they do suggest that the mechanism is different than that of PCA reactions, which involve exocytosis of amine storage granules (39). MAO inhibition should not affect any response which is mediated solely by granule release because oxidative deamination takes place in the cell cytosol rather than in the granule (22, 23). Reserpine causes 5-HT to leak out of granules into the cytosol where inactivation by MAO takes place (22, 23, 38, 45). Thus, MAO inhibition in theory should only protect reserpine-inhibited reactions in which the cytosol as well as the granules is released. Since MAO inhibition partly protected the delayed response of mice treated with reserpine and failed to protect the PCA response, it seems probable that mast cell cytosol is released in delayed reactions. Cytosol release would occur if the mast cell was killed or damaged by the T cell. T cells of appropriately immunized mice are known to migrate to the skin and to release cytotoxic factors in the presence of specific antigen (2, 46). These released factors are toxic for macrophages and may be related to the lymphokine called lymphotoxin (47). If lymphotoxin was toxic for mast cells it could be the important link between the T cell and the firing of the mast cell. A number of points raised in the above hypothesis are easily testable. Some of them have already been confirmed and others we are in the process of testing.

Several remarks of clarification are in order. Although all the evidence we have presented suggests that 5-HT is the main vasoactive amine involved in the mediation of the reactions of DTH in the mouse [as it is in anaphylactic reactions (16)], we wish to stress that participation by other amines or factors has not been ruled out. There is ample evidence to indicate that different vasoactive amines can interact with one another in the production of vascular alterations (48). In addition, the role of 5-HT carrying platelets (49) in these reactions must be considered. Clearly, a great deal more work is required before the chemical basis for our observations can be soundly and thoroughly elucidated. The present report serves simply to implicate the importance of vasoactive amine release in DTH. This fact has heretofore not been fully appreciated, although the early work of Voisin was highly suggestive in that it showed that vascular permeability changes occurred during the evolution of DTH reactions (50). Perhaps one reason the establishment of the role of vasoactive amines in DTH has been delayed is that the amines and the interactions between them and their various cell receptors are so complex. For example, the nature of histamine (26) and serotonin (24, 25) receptor subtypes has only been recently recognized. The mouse is probably an exceptional model for these studies since histamine mediation of vascular reactions in this species is so much less important than in others (16), allowing us to manipulate the response by affecting the storage of 5-HT. Obviously, we must try to duplicate our results in other species but, from our preliminary attempts to do so in the guinea pig, it seems that this will be a more formidable task than it was in the mouse. We should also point out that there have been a number of reports on the effect of reserpine on the immune response (see 51). These previous studies, however, all used multiple injections of reserpine, which led to cachexia and stress; therefore it is difficult to relate them to the findings we have made.

Finally, we would like to comment on what relationship our findings may

have to other areas of cellular immunology such as tumor rejection. It has been shown that some tumors can elicit a very strong cell-mediated immune response against themselves and yet continue to grow progressively in the face of this immune response until death of the host; a phenomenon referred to as concomitant immunity (52). Although the primary tumor is not rejected, subcutaneous or intravenous grafts of isolated tumor cells fail to take in the animal with the progressively growing tumor. In addition, the concomitant immunity is able to prevent viable circulating tumor cells in the tumor-bearing host from forming metastatic deposits. Although quantitative factors are certainly involved, it has been shown that, over and above these, the tumor acquires resistance to the hosts immune response when it becomes stromatized (53). We suggest that a possible mechanism accounting for the phenomenon of concomitant immunity is that tumors may lack (*a*) mast cells, or (*b*) postcapillary venules and thus may be isolated to some degree from the immune killer cells they have engendered in the host in which they are growing. A parallel situation has been reported in experiments on allergic encephalomyelitis (54, 55).

The work we have presented highlights an important, under-investigated area of immunology: delivery of cells to sites of reaction. The finding that tumor-bearing hosts contain lymphocytes which are able to kill tumor cells in vitro but which have not been demonstrated to have in vivo effects, indicates that the problem of delivery may be of broader importance in health care then has heretofore been generally realized.

Summary

The skin sites of the mouse where delayed-type hypersensitivity (DTH) reactions are most easily elicited (foot pads and ears) are particularly rich in 5-hydroxytryptamine (5-HT)-containing mast cells. Since mice are deficient in circulating basophils, which play a role in at least some DTH reactions, we investigated the possibility that the mast cells were playing an important role in the evolution of the skin reactions of DTH in mice. We found that reserpine, a drug which depletes mast cells of 5-HT, abolished the ability of the mouse to make DTH reactions in the skin. The suppressive effect of reserpine could be partially blocked by monoamine oxidase inhibitors which prevent the degradation of 5-HT in the cytosol of the mast cell. Spleen cells of immune, reserpine-treated mice transferred DTH reactions to nonimmune mice normally, indicating that the reserpine treatment did not affect immune T cells. DTH reactions could not be transferred into reserpine-treated mice.

We suggest that T cells are continually emigrating from the blood, through postcapillary venule endothelium, by a mechanism which does not depend on vasoactive amines. If they are appropriately immune and meet the homologous antigen in the tissue, they induce mast cells to release vasoactive amines which cause postcapillary venule endothelial cells to separate, allowing the egress from the blood of cells which ordinarily do not recirculate. The secondarily arriving vasoactive amine-dependent cells are responsible for the micro- and macroscopic lesions of DTH reactions. Chemotactic factors may also be involved in bringing cells to the DTH reaction sites but we propose that T-cell regulation of vasoactive amine-containing cells allows the effector cells to pass through the endothelial gates after they are called.

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Bibliography

1. Munoz, J. 1967. Immediate hypersensitivity reactions induced in mice by active and passive means. *J. Immunol.* 98:638.
2. Gershon, R. K., and R. S. Hencin. 1971. Production of delayed hypersensitivity-type lesions in the flank skin of mice. *In* Cellular Interactions in the Immune Response. S. Cohen, G. Cudkowicz, and R. T. McCluskey, editors. Karger AG, Basel. 207.
3. Crowle, A. J. 1975. Delayed hypersensitivity in the mouse. *Adv. Immunol.* 20:197.
4. Gray, D. F., and P. S. Jennings. 1955. Allergy in experimental mouse tuberculosis. *Am. Rev. Tuberc. Pulm. Dis.* 72:171.
5. Asherson, G. L., and W. Ptak. 1968. Contact and delayed-hypersensitivity in the mouse. *Immunology.* 15:405.
6. Waksman, B. H. 1960. A comparitive histopathological study of delayed hypersensitive reactions. *In* Cellular Aspects of Immunity. Ciba Foundation Symposium. G. E. W. Wolstenholme and M. O'Connor, editors. J. and A. Churchill Ltd., London. 280.
7. Kosunen, T. U., B. H. Waksman, M. H. Flax, and W. S. Tihen. 1963. Radioautographic study of cellular mechanisms in delayed hypersensitivity. I. Delayed reactions to tuberculin and purified protein in the rat and guinea pig. *Immunology.* 6:276.
8. Najarian, J. S., and J. D. Feldman. 1963. Specificity of passively transferred delayed hypersensitivity. *J. Exp. Med.* 118:341.
9. McCluskey, R. T., B. Benacerraf, and J. W. McCluskey. 1963. Studies on the specificity of the cellular infiltrate in delayed hypersensitivity reactions. *J. Immunol.* 90:466.
10. Cohen, S., R. T. McCluskey, and B. Benacerraf. 1967. Studies on the specificity of the cellular infiltrate of delayed hypersensitivity reactions. *J. Immunol.* 98:269.
11. Lubaroff, D. M., and B. H. Waksman. 1968. Bone marrow as source of cells in reactions of cellular hypersensitivity. I. Passive transfer of tuberculin sensitivity in syngeneic systems. *J. Exp. Med.* 128:1425.
12. Dvorak, H. F., and A. M. Dvorak. 1974. Cutaneous basophil hypersensitivity. *In* Progress in Immunology II. Vol. 3. L. Brent and J. Holborrow, editors. North-Holland Publishing Co., Amsterdam. 171.
13. DeBernardo, R., P. Askenase, D. Tauben, and J. Douglas. 1975. Augmented anaphylaxis at sites of cutaneous basophil hypersensitivity (CBH). *J. Allergy Clin. Immunol.* 55:111.
14. Selye, H. 1965. The Mast Cell. Butterworth Inc., Washington, D.C.
15. Fink, M. A. 1956. Anaphylaxis in the mouse: possible relation of the Schultz-Dale reaction to serotonin release. *Proc. Soc. Exp. Biol. Med.* 92:673.
16. Gershon, M. D., and L. L. Ross. 1961. Studies on the relationship of 5-hydroxytryptamine and enterochromaffin cell to anaphylactic shock in mice. *J. Exp. Med.* 115:367.
17. Majno, G., G. E. Palade, and G. I. Schoefl. 1961. Studies on inflammation II. The site of action of histamine along the vascular tree: a topographic study. *J. Biophys. Biochem. Cytol.* 11:607.
18. Phanuphak, P., J. W. Moorhead, and H. N. Claman. 1974. Tolerance and contact sensitivity to DNFB in mice. *J. Immunol.* 112:115.
19. Kettman, J. 1972. Delayed hypersensitivity: is the same population of thymus derived cells responsible for cellular immunity reactions and the carrier effect? *Immunol. Comm.* 1:289.

20. Ovary, Z. 1958. Immediate reactions in the skin of experimental animals provoked by antibody-antigen interreaction. *Prog. Allergy.* 5:459.
21. Askenase, P. W., and B. J. Hayden. 1974. Cytophilic antibodies in mice contact-sensitized with oxazolone—immunochemical characterization and preferential binding to a trypsin-sensitive macrophage receptor. *Immunology.* 27:563.
22. Shore, P. A. 1962. Release of serotonin and catecholamines by drugs. *Pharmacol. Rev.* 14:531.
23. Carlsson, A. 1966. Drugs which block the storage of 5-hydroxytryptamine and related amines. *In* Handbook of Experimental Pharmacology. 5-Hydroxytryptamine and Related Indolealkylamines. Springer-Verlag, New York. 529.
24. Drakontides, A. B., and M. D. Gershon. 1968. 5-Hydroxytryptamine receptors in the mouse duodenum. *Br. J. Pharmacol. Chemother.* 33:480.
25. Gerschenfeld, H. M., and D. Paupardin-Tritsch. 1974. Ionic mechanisms and receptor properties underlying the responses of molluscan neurones to 5-hydroxytryptamine. *J. Physiol. (Lond.).* 243:427.
26. Wyllie, J. H., T. Hesselbo, and J. W. Black. 1972. Effects in man of histamine H_2-receptor blockade by burimamide. *Lancet.* 2:117.
27. Malmfors, T., and H. Thoenen, editors. 1971. 6-Hydroxydopamine and catecholamine neurons. North-Holland Publishing Co., Amsterdam.
28. Gershon, M. D., and R. F. Altman. 1971. An analysis of the uptake of 5-hydroxytryptamine by myenteric plexus of the small intestine of the guinea pig. *J. Pharmacol. Exp. Ther.* 179:29.
29. Gershon, M. D., and L. L. Ross. 1966. Radioisotopic studies of the binding, exchange, and distribution of 5-hydroxytryptamine synthesized from its radioactive precursor. *J. Physiol. (Lond.).* 186:451.
30. Gershon, M. D., and L. L. Ross. 1966. Location of sites of 5-hydroxytryptamine storage and metabolism by radioautography. *J. Physiol. (Lond.).* 186:477.
31. Fischman, D., and M. D. Gershon. 1964. A method for studying intracellular movement of water soluble isotopes prior to radioautography. *J. Cell. Biol.* 21:139.
32. Flack, B., and C. Owman. 1965. A detailed methodological description of the fluorescence method for the cellular demonstration of biogenic monoamines. *Acta Univ. Lund. Sect. II Med. Math. Sci. Reum. Nat.* 7:1.
33. Fuxe, K., and G. Jonsson. 1967. A modification of the histochemical fluorescence method for the improved localization of 5-hydroxytryptamine. *Histochemie.* 11:161.
34. Ehinger, B., and R. Thunberg. 1967. Induction of fluorescence in histamine-containing cells. *Exp. Cell Res.* 47:116.
35. Cross, S. A. M., S. W. B. Ewen, and F. W. D. Rost. 1971. A study of the methods available for the cytochemcal localization of histamine by fluorescence induced with *o*-phalaldehyde or acetaldehyde. *Histochem. J.* 3:471.
36. Askenase, P. W. 1973. Cutaneous basophil hypersensitivity in contact-sensitized guinea pigs. I. Transfer with immune serum. *J. Exp. Med.* 138:1144.
37. Phair, J. P., A. J. Eisenfeld, R. J. Levine, and F. S. Kantor. 1970. Effects of pharmacological inhibition of histamine synthesis upon immunological reactions in guinea-pigs. *Immunology.* 18:611.
38. Van Orden, L. S., I. Vugman, K. G. Bensch, and N. J. Giarman. 1967. Biochemical, histochemical and electron-microscopic studies of 5-hydroxytryptamine in neoplastic mast cells. *J. Pharmacol. Exp.* 158:195.
39. Anderson, P., S. A. Slorach, and B. Unas. 1973. Sequential exocytosis of storage granules during antigen-induced histamine release from sensitized rat mast cells *in vitro*. An electronmicroscopic study. *Acta. Physiol. Scand.* 88:359.
40. Miller, J. F. A. P., and G. F. Mitchell. 1969. Thymus and antigen-reactive cells. *Transplant. Rev.* 1:3.

41. Zatz, M. M., and R. K. Gershon. 1974. Thymus dependence of lymphocyte trapping. *J. Immunol.* **112**:101.
42. David, J. R., and R. A. David. 1972. Cellular hypersensitivity and immunity. Inhibition of macrophage migration and the lymphocyte mediators. *Prog. Allergy.* **16**:300.
43. Marchesi, V. T., and J. L. Gowans. 1964. The migration of lymphocytes through the endothelium of venules in lymph nodes: an electron microscope study. *Proc. Roy. Soc. Lond. B. Biol. Sci.* **159**:283.
44. Schoefl, G. I. 1972. The migration of lymphocytes across the vascular endothelium in lymphoid tissue: a reexamination. *J. Exp. Med.* **136**:568.
45. Hollenbeck, R. A., and P. A. Shore. 1974. Localization and binding of reserpine in the membrane of adrenal medullary amine storage granules. *Naunyn-Schmiedebergs Arch. Exp. Pathol. Pharmakol.* **283**:263.
46. Nomoto, K., R. K. Gershon, and B. H. Waksman. 1970. The role of non-immunized macrophages in rejection of an allo-transplantable lymphoma in the hamster. *J. Natl. Cancer Inst.* **44**:739.
47. Ruddle, N. H., and B. H. Waksman. 1968. Cytotoxicity mediated by soluble antigen and lymphocytes in delayed hypersensitivity. *J. Exp. Med.* **128**:1237.
48. Gozsy, B., and L. Kato. 1966. Role of norepinephrine and 5-hydroxytryptamine in the delayed phase of the inflammatory reaction in rats. *Int. Arch. Allergy Appl. Immunol.* **30**:553.
49. Osler, A. G., and P. R. Siraganian. 1972. Immunologic mechanisms of platelet damage. *Prog. Allergy.* **16**:450.
50. Voisin, G. A., and F. Toullet. 1960. Modifications of capillary premeability in immunological reactions mediated through cells. *In* Cellular Aspects of Immunity. Ciba Foundation Symposium. G. E. W. Wolstenholme and M. O'Connor, editors. J. and A. Chruchill Ltd., London. 373.
51. Dukor, P., S. B. Salvin, F. M. Dietrich, J. Gelzer, R. Hess, and P. Loustalot. 1966. Effect of reserpine on immune reactions and tumor growth. *Eur. J. Cancer.* **2**:253.
52. Gershon, R. K., R. L. Carter, and K. Kondo. 1967. Observations on the nature of concomitant immunity in tumor-bearing hamsters. *Nature (Lond.).* **213**:674.
53. Gershon, R. K. 1974. Regulation of concomitant immunity: activation of suppressor cells by tumor excision. *Isr. J. Med. Sci.* **10**:1012.
54. Clark, G., and L. H. Bogdanove. 1955. The induction of the lesions of allergic meningoencephalomyelitis in a predetermined location. *J. Neuropathol. Exp. Neurol.* **14**:433.
55. Bogdanove, L. H., and G. Clark. 1957. The induction of exacerbations of allergic meningoencephalomyelitis. *J. Neuropathol. Exp. Neurol.* **16**:57.