

CUTANEOUS BASOPHIL HYPERSENSITIVITY IN CONTACT-SENSITIZED GUINEA PIGS

I. TRANSFER WITH IMMUNE SERUM*

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Dvorak and co-workers have recently drawn attention to an entirely new class of hypersensitivity reaction characterized by infiltrates of basophils (1, 2). In guinea pigs, this cutaneous basophil hypersensitivity (CBH)¹ is delayed in time-course but unlike classical delayed type (tuberculin) sensitivity (DH) the basophil-containing reactions show less induration and are not associated with the production of macrophage migration inhibition factor (3).

CBH has been related to Jones-Mote reactions (4, 5), originally described in man, and accumulations of basophils have been noted in patients with contact dermatitis (6). In guinea pigs dense infiltrates of basophils have been demonstrated in: contact reactions (7), immunity to viruses (8) and ticks (9), allograft rejection (10), and tumor immunity (11). Thus basophil hypersensitivity appears to be a widely occurring immune inflammatory response.

The crucial demonstration of basophils in these reactions has until now required the elaborate tissue preparation techniques of electron microscopy. In the experiments reported here basophil hypersensitivity was demonstrated by simple and inexpensive histological procedures employing paraffin-embedded material.

CBH is generally elicited in animals immunized without mycobacterial adjuvants, while DH requires the use of these substances (1, 3, 12, 13). However, Dvorak et al. (7) showed that animals immunized for DH with complete Freund's adjuvant (CFA) and challenged at 1 wk had skin reactions containing substantial infiltrates of basophils, while reactions elicited later after sensitization were more indurated and contained insignificant numbers of basophils. They concluded that classical "delayed hypersensitivity included a basophilic component early in its evolution that diminished with time." This report de-

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¹ Abbreviations used in this paper: CBH, cutaneous basophil hypersensitivity; CFA, Freund's complete adjuvant; DH, delayed hypersensitivity; Ox-KLH, oxazolone keyhole limpet hemocyanin; PBS, phosphate-buffered saline; PPD, purified protein derivative of tuberculin.

scribes the transfer of CBH with serum from contact-sensitized guinea pigs and shows that basophil hypersensitivity found in animals immunized with conjugates in CFA is a hapten-specific delayed-in-time reaction that can also be transferred with immune serum.

Materials and Methods

Animals.—Outbred male Hartley strain albino guinea pigs (Perfection Breeders, Douglassville, Pa.), weighing about 450 g were used in all experiments.

Antigens.—An ammonium sulfate precipitate of keyhole limpet hemocyanin (KLH) (Pacific Biomarine Supply Co., Venice, Calif.) was dissolved by dialysis against pH 8.5 sodium phosphate buffer. Insoluble material was removed by centrifugation. The protein concentration was adjusted to 20 mg/ml and 5% sodium carbonate was added until the pH was 8.5. 1% oxazolone (2-phenyl-4-ethoxymethylene oxazolone) (Gallard-Schlesinger Chemical Mfg. Corp., Carle Place, N. Y.) in ethanol (0.5 ml) was rapidly added to 10 ml of the KLH solution on a magnetic stirrer, and stirring was maintained for 1 h at room temperature. Glycine was then added to a final concentration of 0.2 M to neutralize any remaining reactive hapten; stirring was continued for another hour, insoluble material was again removed by centrifugation, and the conjugated protein was freed of low molecular weight material by gel filtration chromatography using Sephadex G25 (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.) equilibrated with 5 mM phosphate-buffered 0.15 M saline (PBS). Protein determination was by micro-Kjeldahl and 1 M oxazolone was assumed to give 18,400 OD units absorbance at 352 nm (14). The two preparations of oxazolone-KLH used in this study were found to contain 1,270 and 1,100 haptenic groups per molecule of protein.

Immunization.—Protein antigens (100 μ g per animal) in PBS were emulsified with Freund's complete adjuvant (CFA-H37Ra) (Difco Laboratories, Detroit, Mich.) (containing 3 mg/ml ground *Mycobacterium tuberculosis*) and were injected into each footpad (0.1 ml) on day 0. Contact sensitization was accomplished by daily painting of the shaved abdomen or back with 0.5 ml of 12% oxazolone in ethanol on days 0-4. In one experiment animals were contact boosted with a single painting of 4% oxazolone (1.0 ml) on day 10.

Macroscopic Quantification of Contact Skin Reactions.—Oxazolone (4% in olive oil) was applied to the ears of previously sensitized animals and increase in ear thickness was measured with a micrometer (Brown and Sharpe Mfg. Co., N. Kingston, R. I.) (15). In experiments dealing with the specificity of contact reactions, 4% oxazolone was applied to one ear and 4% picryl chloride to the other ear. Before this contact challenge, guinea pig ear edge thickness at a marked spot (Drimark Products, Inc., Mt. Vernon, N. Y.) averaged about 0.6 mm (± 0.007 mm). Thus a 25% change represented about 0.15 mm increase in ear thickness and a +10% change was usually statistically significant ($P < 0.05$ Student's *t* test).

Epon Embedding for Light and Electron Microscopy.—24-h skin reactions were excised, cut into 1-2 mm strips, fixed in 5% phosphate-buffered (pH 7.3) glutaraldehyde (Fisher Scientific Co., Pittsburgh, Pa.), embedded in Epon 812 (Ladd Research Industries Inc., Burlington, Vt.), cut into 1-3 μ m sections, and stained with Giemsa (2). Observation by light microscopy revealed lesions typical of basophilic hypersensitivity and parallel observation by electron microscopy showed the characteristic granules of the infiltrating basophils (2).

Paraffin Embedding for Routine Histology.—Ears were excised, cut into strips, and placed in several histological fixatives. Tissues placed in Helly's fixative (100 ml Zenker's solution (Fisher Scientific Co.) + 5 ml 40% formaldehyde), embedded in paraffin, cut in 3-5 μ m sections, and stained with Giemsa (16), showed basophil infiltrates (Fig. 1) comparable with those seen in the Epon-embedded material. Moreover, basophils could be easily distinguished from eosinophils in the paraffin-embedded sections. In addition, much larger individual specimens and tissue from several animals could be placed in a single block.

Microscopic Quantification of Basophil-Containing Infiltrates.—Slides were coded and baso-

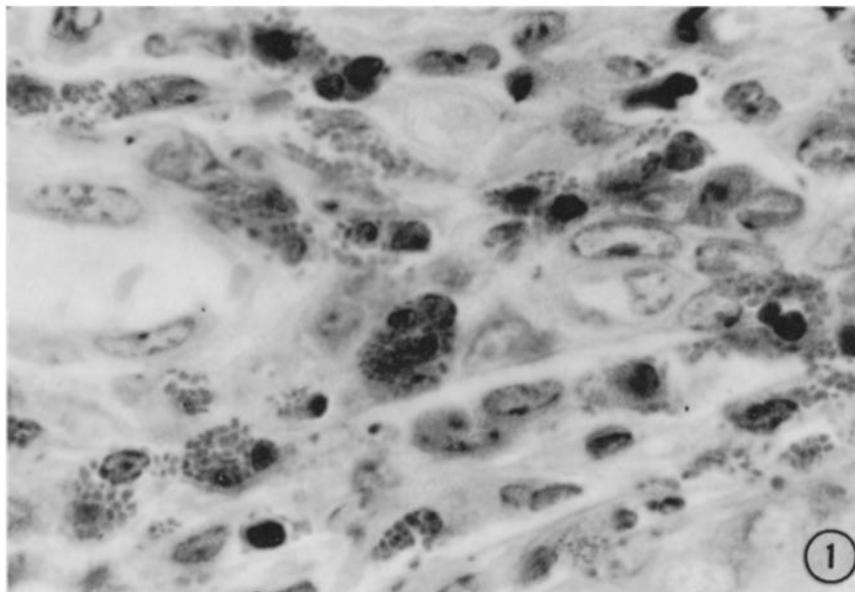


FIG. 1. Light photomicrograph of a Giemsa-stained paraffin section from the ear of a contact-sensitized guinea pig challenged with oxazolone in olive oil. A heavy infiltrate of basophils is seen in an area of dermis just below the dermal-epidermal junction. $\times 1,250$.

phil counts were made near the ear tip in five oil power ($\times 1,000$) fields at the dermal-epidermal junction.

RESULTS

Macroscopic Contact Reactions in Actively Sensitized Guinea Pigs (Table I).— In the first experiment controls were immunized with CFA and the experimental animals were contact sensitized or received CFA emulsified with 100 μg oxazolone-KLH ($\text{Ox}_{1270}\text{KLH}$). 8 days after initial sensitization, animals were challenged by applying 4% oxazolone in olive oil to their ears. Reactions were quantitated by comparing ear thickness measured 24 h after challenge with ear thickness determined before challenge. Table I shows that 4% oxazolone caused no swelling in the ears of unimmunized animals or controls immunized with CFA (group A). After oxazolone challenge there was an obvious increase in ear thickness and erythema in the actively contact-sensitized animals (group B), and in guinea pigs immunized with $\text{Ox}_{1270}\text{KLH}$ in CFA (group C).

In a second experiment (Table II) controls immunized with 100 μg KLH in CFA showed no response to 4% oxazolone while animals sensitized with 100 μg of a second preparation of Ox-KLH conjugate ($\text{Ox}_{1100}\text{KLH}$) had no reactions at 4 h and again had significant increase in ear thickness 24 h after challenge with oxazolone. This delayed reaction in contact-challenged ears of animals sensitized

TABLE I
Skin Reactions of Guinea Pigs Challenged 8 Days after Sensitization

Immunization (no. animals)	24-h skin reaction in ears challenged with 4% oxazolone	
	Ear thickness change \pm SEM*	Basophils per 5 oil power fields \pm SEM
	%	
None (3)	-4 \pm 1.9	5 \pm 1.3
A. CFA (6)	0 \pm 1.6	3 \pm 1.2
B. Oxazolone painting days 0-4 (6)	43 \pm 5 \ddagger	95 \pm 20 \S
C. Ox ₁₂₇₀ KLH in CFA (6)	32 \pm 5.6 \ddagger	51 \pm 5 \S

* Standard error of the mean.

\ddagger $P < 0.005$.

\S $P < 0.0005$.

TABLE II
Specificity of Skin Reactions in Guinea Pigs Challenged 8 Days after Sensitization

Immunization (no. animals)	Skin reactions in contact challenged ears					
	Ear thickness change \pm SEM*				Basophils per 5 oil power fields \pm SEM at 24 h	
	4 h		24 h		Oxazolone	Picryl chloride
	Oxazolone \ddagger	Picryl chloride	Oxazolone	Picryl chloride	Oxazolone	Picryl chloride
	%		%			
A. KLH in CFA(4)	-8	-8	-1	-1	9 \pm 5	5
B. Ox ₁₁₀₀ KLH in CFA(4)	-6	-9	+15 \pm 5.6 \S	-10	96 \pm 20 \S	14

* Standard error of the mean.

\ddagger Each animal had the right ear challenged by application of 4% oxazolone and the left ear challenged by application of 4% picryl chloride.

\S $P < 0.01$.

with Ox-KLH in CFA was interpreted as a hapten-specific response because (a) measures were taken to eliminate residual reactive hapten from the two preparations of Ox-KLH (see Methods); (b) KLH is presumed to represent a carrier distinct from guinea pig proteins serving as carriers in contact painting; and (c) no increase in ear thickness occurred in contralateral ears challenged with 4% picryl chloride (Table II, group B).

Histology of Contact Reactions in Actively Sensitized Guinea Pigs.—Ears from all animals were excised at 24 h and prepared for microscopic examination by the histological techniques outlined above. Basophil counts were made in five oil power fields per paraffin section and are shown in Tables I and II. Ears of unimmunized animals and CFA (Table I), or CFA + KLH (Table II), immunized controls, that had shown no macroscopic swelling, had a nonspecific infiltrate of mononuclear cells and less than two basophils per oil power field, after challenge with oxazolone or picryl chloride.

Contact-sensitized animals (Table I, group B) showed the expected basophilic infiltrate (Fig. 1). Ears of guinea pigs immunized with O_x-KLH that macroscopically had demonstrated hapten-specific delayed-in-time reactions showed a microscopic infiltrate consisting of substantial numbers of basophils in ears challenged with oxazolone. The specificity of this reaction was also evident at the microscopic level, as insignificant numbers of basophils accumulated in ears challenged with picryl chloride (Table II, group B). It was concluded that, in the system under study, CBH was a hapten-specific delayed-in-time reaction.

Serum Transfers.—The finding that CBH was hapten specific raised the possibility that serum factors might participate in this reaction. Accordingly, 3.5–5 ml of serum from actively sensitized donors (Table I and II) were transferred intravenously to normal recipient guinea pigs. In the initial transfer experiment (Table III) donors were immunized with: (A) CFA, (B) multiple oxazolone paintings, or (C) O_x₁₂₇₀KLH in CFA. Donors were then challenged (ear test) on day 8 and bled out on day 9. Another group of donors received contact paintings on days 0–4, were additionally contact boosted (see Methods) on day 10, challenged on day 21, and bled out on day 22 (group D).

1 h after transfer, the recipients were challenged by applying 4% oxazolone in olive oil to their ears. Reading of ear thickness and histology were performed blind. Table III shows that no recipients had significant ear swelling at 4 h. At 24 h after challenge, significant increases in ear thickness were transferred in two of three groups receiving immune serum. Histological examination of these transferred 24-h macroscopic reactions revealed significant infiltrates of basophils (Table III) with a pattern similar to that seen in CBH lesions of the donors.

TABLE III
Passive Transfer of Basophil-Containing Skin Reactions with Immune Serum

Immunization of Donors (no. recipients)	Contact reactions in the ears of recipients challenged with oxazolone		
	Ear thickness 4 h	change \pm SEM* 24 h	Basophils per 5 oil power fields \pm SEM at 24 h
	%	%	
A. CFA (3)‡	–2	+1 \pm 3.8	3 \pm 0.1
B. Oxazolone painting days 0–4 (3)‡	–4	+6 \pm 3	84 \pm 38§
C. O _x ₁₂₇₀ KLH in CFA (3)‡	–2	+16 \pm 1.7	45 \pm 18§
D. Oxazolone painting days 0–4 and 10 (3)¶	–1	+36 \pm 5.9**	49 \pm 17

* Standard error of the mean.

‡ Donors challenged on day 8 after immunization and bled out on day 9.

§ $P < 0.05$.

|| $P < 0.025$.

¶ Donors boosted on day 10, challenged on day 21, and bled on day 22.

** $P < 0.005$.

An additional observation was made in the immune serum recipients which failed to demonstrate the transfer of any macroscopic reactions (Table III, group B). At 24 h after challenge, ears from these recipients also showed a heavy infiltrate of basophils in paraffin sections (Fig. 2), and in Epon-embedded material viewed by light (Fig. 3) and by electron microscope (Fig. 4). Thus, delayed-in-time reactions characterized by dense infiltrates of basophils were obtained in three different groups of immune serum recipients, and one of these recipient groups had shown no detectable gross lesions. It was possible that transfer of serum from immunized donors per se had led to a nonspecific basophil-containing infiltrate upon challenge with the high concentrations of contactant used in these experiments. However, transfer of serum from donors immunized with CFA alone (Table III, group A) failed to cause the accumulation of basophils in similarly challenged ears.

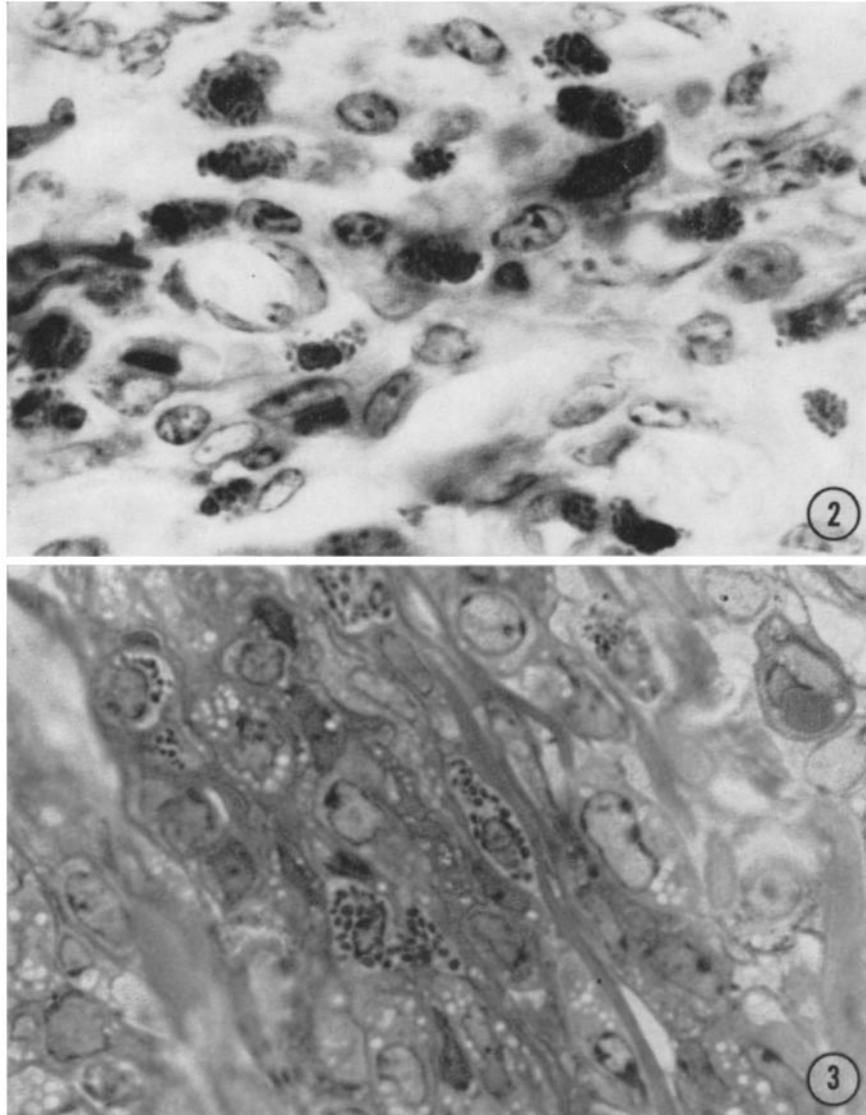
Specificity of Transferred Reactions.—Serum transfers to larger groups of recipients were repeated using donors from Table II. The specificity of transfer was tested by challenging one ear of recipients with oxazolone and the other ear with picryl chloride. Sera from donors immunized with Ox-KLH in CFA again transferred a delayed-in-time basophil-containing infiltrate to the ears of recipients challenged with oxazolone (Table IV). No ear thickening or basophil infiltration was observed in ears of serum recipients challenged with picryl chloride (Table IV, groups B and B').

The Effect of Donor Challenge on Serum Transfer.—Since transfers were from recently challenged donors, it seemed possible that challenge was contributing to the successful serum transfers. However, when animals were immunized with Ox-KLH in CFA and not challenged, serum obtained at day 9 transferred CBH to six of six recipients (Table IV, group B'). In all, 18 of 21 recipients of oxazolone immune sera transferred specific delay-in-time reactions containing dense infiltrates of basophils.

DISCUSSION

The experiments reported here show that the characteristic infiltrate of cutaneous basophil hypersensitivity can be demonstrated by techniques available in any routine histological laboratory. In paraffin sections fixed in Helly's solution and stained with Giemsa, the granules of basophils were preserved and appeared large and dark blue. This allowed for easy distinction of these cells from eosinophils and polys. The techniques used in this study also have demonstrated that significant infiltrates of eosinophils may accompany the basophils. This will be the subject of a future communication.

This study has verified that contact reactions contain dense infiltrates of basophils. Although earlier reports emphasized that CBH was found in guinea pigs immunized without mycobacterial adjuvants, I have confirmed the finding of Dvorak et al. (7) that immunization with CFA can also result in basophil-containing reactions. In addition, it has been shown that animals sensitized



FIGS. 2 and 3. Light photomicrographs of Giemsa-stained sections from the ears of a guinea pig that received a transfer of immune serum from contact-sensitized donors and failed to show a macroscopic reaction after challenge with oxazolone. One excised ear was prepared for routine paraffin section (Fig. 2) and the other for Epon-embedded section (Fig. 3). A dense infiltrate of basophils is evident in Fig. 2 and verified in Fig. 3. $\times 1,250$.

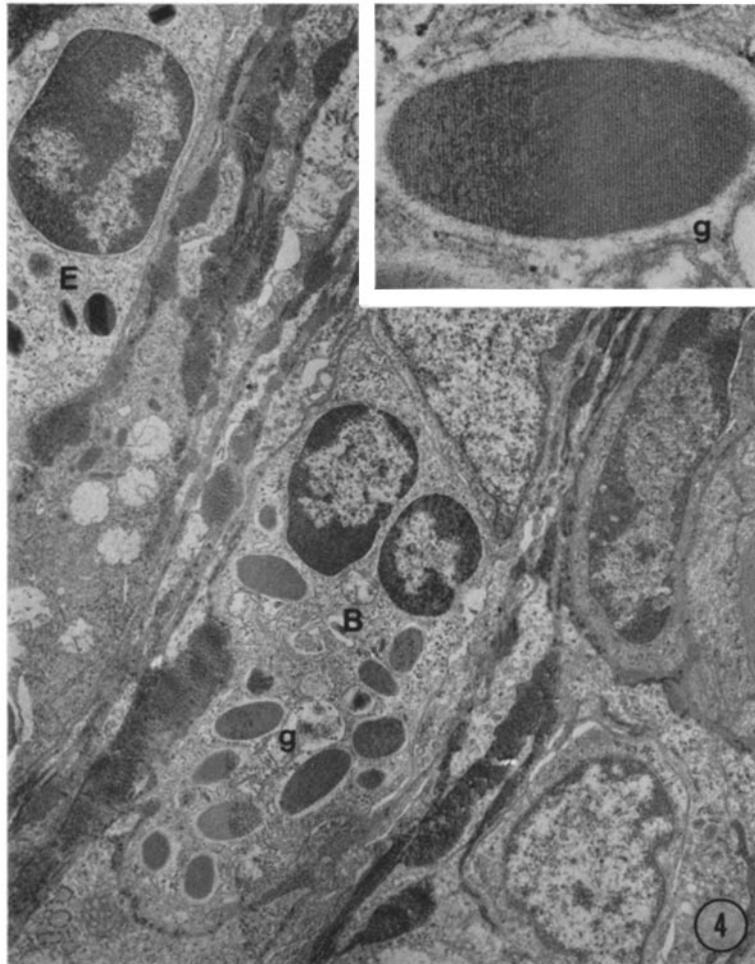


FIG. 4. Electron photomicrograph of an Epon-embedded section from the ear of a guinea pig demonstrating serum transfer of cutaneous basophil hypersensitivity. A basophil (*B*) is seen in the dermis as well as a portion of an eosinophil (*E*), above and to the left. $\times 5,500$. *Insert* contains a higher magnification of a granule (*g*) from the basophil showing the characteristic parallel banded pattern. $\times 39,000$.

with conjugates of hapten and heterologous carrier emulsified with CFA, can demonstrate strong CBH reactions when contact challenged with high concentrations of hapten. This result may provide a morphological basis for earlier observations of Gell and Benacerraf (17) who showed that carrier specificity of delayed reactions could be overcome by challenge with high concentrations of hapten on another carrier.

TABLE IV
Specificity of Serum Transfer of Basophil-Containing Skin Reactions

Immunization of donors (no. recipients)	Skin reactions in contact-challenged ears of recipients					
	Ear thickness change \pm SEM*				Basophils per 5 oil power fields \pm SEM at 24 h	
	4 h		24 h		Oxazolone	Picryl chloride
	Oxazo- lone \ddagger	Picryl chloride	Oxazolone	Picryl chloride		
%	%	%	%			
Challenged donors						
A. KLH in CFA(6) \S	-5	-10	+3 \pm 1.8	-2	1 \pm 0.7	0
B. Ox ₁₁₀₀ KLH in CFA(6) \S	-3	-3	+8 \pm 4	0	22 \pm 7 \parallel	7
Nonchallenged donors						
A. KLH in CFA (6) \P	-2	-6	0 \pm 2	-3	6 \pm 2.5	5
B. Ox ₁₁₀₀ KLH in CFA (5) \P	-6	-8	+10 \pm 2.7**	-6	58 \pm 6.5 $\ddagger\ddagger$	3

* Standard error of the mean.

\ddagger Each animal had the right ear challenged by application of 4% oxazolone and the left ear challenged by application of 4% picryl chloride.

\S Donors challenged on day 8 after immunization and bled out on day 9.

\parallel $P < 0.05$.

\P Donors not challenged, and then bled out on day 9.

** $P < 0.01$.

$\ddagger\ddagger$ $P < 0.0005$.

In the present study it was possible that immunizing hapten-protein conjugates contained small amounts of free contactant leading to active contact sensitization when emulsified with CFA. However, I have confirmed the previous finding of Dvorak et al. (7), that animals intentionally immunized with contactant in CFA and, subsequently contact challenged, have a delayed response that contains fewer basophils and closely resembles a classical delayed reaction elicited by purified protein derivative of tuberculin (PPD) skin testing. Furthermore, the basophil-containing, hapten-specific, delayed reactions of guinea pigs immunized with Ox-KLH in CFA could be transferred with immune serum, while classical delayed skin reactions are not transferred with serum.

This study is the first report of transfer with immune serum of significant macroscopic delayed-in-time reactions featuring infiltrates of basophils. Tables III and IV show that a specific 24-h histological infiltrate was also found in three groups of recipients that had shown minimal macroscopic ear swelling. Thus, newer techniques have demonstrated serum transfer of a specific histological infiltrate in recipients failing to uniformly show macroscopic reactions. This finding supports and may help explain occasional reports of successful serum transfer of weak delayed-in-time reactions (reviewed in 18-21). In the

only previously reported study on the transfer of CBH (7), microscopic lesions were transferred with lymph node cells and not by serum. Reasons for the difference between that study and the findings reported here are not known, but may have been due to differences in the preparation of donors, transfer, or challenge of recipients.

The factors in immune sera responsible for successful transfer are unknown. These sera contained antibodies to oxazolone (22) but some sera with weak titers have produced successful transfers. Class or affinity of antibody may be important. Since the donors were generally challenged 24 h before serum harvest it seemed possible that factors released by sensitized cells might have contributed to successful transfers.² However, Table IV shows that when immune donors were not challenged before bleeding, their harvested sera still transferred CBH (group B'). Thus the factors in immune serum responsible for transfer are not dependent on challenge of the donors (20).

In addition to the marked basophil infiltrate, CBH has been distinguished from classical delayed type (tuberculin) hypersensitivity by several parameters: (a) lack of a need for mycobacterial adjuvants (1, 3, 12, 13); (b) transient duration after sensitization with soluble antigens (1, 7); (c) immunogenic carrier requirements resembling those for antibody (1); and (d) lack of participation by macrophages (1, 3). Additional features distinguishing CBH from DH were found in the experimental system used in this study. Whereas classical delayed reactions are carrier specific and transferable by cells and not serum, basophil hypersensitivity, although delayed in time-course, was found to be hapten specific and transferable by immune serum.

SUMMARY

Cutaneous basophil hypersensitivity, an immune inflammatory reaction characterized by infiltrates of basophils and a delayed time-course, was studied in guinea pigs contact sensitized with oxazolone. Routine histological techniques, employing ordinary paraffin sections, were modified to study this reaction. When biopsies of contact lesions were processed by these methods dense infiltrates of basophils could be demonstrated. Animals sensitized with complete Freund's adjuvant emulsified with oxazolone-keyhole limpet hemocyanin conjugates also developed delayed-in-time responses to contact challenge with oxazolone but not to picryl chloride. These hapten-specific delayed-in-time reactions also contained substantial numbers of basophils. Transfer of serum from actively sensitized guinea pigs resulted in specific accumulation of basophils at challenge sites of recipients. Thus, in this experimental system, cutaneous basophil hypersensitivity was found to be a hapten-specific delayed-in-time reaction that could be transferred with immune serum.

² Boetcher, D. A., and E. J. Leonard. Basophil chemotaxis: augmentation by a factor from stimulated lymphocyte cultures. *Immunological Communications*. In press.

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