The Nature of Late Reactions Following Intradermal Injection of Heterologous Anti-Tissue Sera.* (29232)

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Heterologous antisera to tissues with epithelial and/or mesenchymal basement membranes (B.M.) contain antibodies which on intradermal injection have a striking affinity for the B.M. of skin as demonstrated by immunofluorescence. These antisera produce at the same time a characteristic monophasic inflammatory reaction with acute vasculitis and hemorrhage. It has been proposed that this reaction is due to the combination of specific anti-B.M. antibodies with antigens present in capillary and venular B.M. of either "common" ("non-collagenous" antigens common to all B.M.) or "nephrotoxic" (antigens present in capillary and venular B.M. only) type or both(1,2). It appeared that the combination of these antibodies with the "common" antigens in non-capillary, non-venular B.M. contributed relatively little to the specific acute inflammatory reaction.

Heterologous antisera to tissues devoid of B.M. such as the substantia propria of the cornea or cartilage produce on intradermal injection a characteristic biphasic type of reaction with a diffuse polymorphonuclear leukocytic reaction in the earlier phase and a massive monocytic-histiocytic infiltration in the later. It was assumed that these reactions were due to the combination of specific antibodies with antigens in the ground substance or the non-fibrillar mesenchymal matrix of skin. Antibodies were present in these antisera which were bound *in vivo* to the B.M. of skin, but in all, such binding as could be demonstrated by immunofluorescence was not as marked or as regular as with anti-B.M. antibodies. It was felt that these antibodies were related to a third or "collagenous" antigen, presumably present in all B.M. and that their union with these antigens played a subordinate role in the overall inflammatory response(1,2).

In following the dermal reactions to anti-tissue sera beyond the first 72 hours, late reactions were noted which differed from the initial acute ones. The nature of these lesions will be described and their relationship to heterologous antibodies fixed to the B.M. of skin will be demonstrated.

Materials and methods. Rats of the Holtzman strain were used as a source of tissue, and albino rabbits for production of antisera. The preparation of antisera to glomerular B.M., cartilage, tail tendon, corneal epithelium, substantia propria of the cornea, lens capsule, nuclear portion of lens and vitreous humor has been described(1,2). Fresh intact liver cells were prepared by sieving fragments of liver through a 150 mesh monel-metal screen with the convex portion of a sterile spoon. The sievings were washed repeatedly by centrifugation with 0.15 M saline until the supernatant was clear. The sediment was then resuspended in saline and by means of a brief and rapid spin in the centrifuge intact cells were carried to the bottom of the tube. The fragmented cells in the supernatant were withdrawn by suction. Rabbits were injected intramuscularly with the following wet weights of intact cells suspended in aluminum jelly according to the method of Tracy and Welker(3) and at the stated intervals: 150 mg initially; 300 mg at 7 days; 450 mg at 17 days; 600 mg at 38 days; 750 mg at 48 days and 900 mg at 55 days. To prepare antisera to rat serum, packed rat red blood cells and rat platelets resp., the rabbits were given daily intravenous injections of 0.1 ml of each per rabbit to a total of 1.0 ml. In the case of platelets, 2 additional injections of 0.1 ml in aluminum jelly were given intramuscularly on the second and sixth days.

The agglutination titer of the anti-rat red blood cell serum against rat red blood cells was 1:4096 and that of the anti-rat platelet serum against rat platelets 1:512. The precipitin titer of the anti-rat serum against rat serum was 1:16,000 and of anti-vitreous

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humor against vitreous humor 1:8. Owing to the physical state and insolubility of the antigens of the other tissue components, the potency of their antisera was determined periodically in the course of the immunizations by the quality of the reactions obtained on intradermal injection of rats. The most active sera were used in this study.

The rabbits were bled 10 days after the last injection. Serum was withdrawn and stored at —15°C. All sera prior to use were inactivated at 56°C for a half hour and except for the anti-rat red blood cell serum were absorbed repeatedly with fresh washed rat red blood cells.

The antisera to cartilage, substantia propria of the cornea and tendon resp., were absorbed 3 times with sonically vibrated lens capsule using 1.0 g/ml of antiserum/absorption and 2 to 3 times with glomerular B.M. using 200 mg/ml of antiserum/absorption. These antisera were also absorbed with their non-homologous sonically vibrated tissues using 1.0 g wet weight of tissue/ml of antiserum/absorption viz., anti-cartilage serum 5 times with cornea and with a second batch 8 times with tendon, anti-cornea serum 6 times with cartilage and with a second batch 8 times with tendon, anti-tendon serum 4 times with cartilage and with a second batch 4 times with cornea. The antiserum to glomerular B.M. was absorbed 3 times with sonically vibrated myocardium and with a second batch 3 times with sonically vibrated lung using 1.0 g of wet tissue/ml of antiserum/absorption.

Each absorption was carried out on a mechanical shaker by incubation of the mixture at 37°C for 1 hour and subsequently at 2°C for 18 hours. The absorbed serum was removed following centrifugation of the mixture in a Servall at 18,000 rpm for 30 minutes at 2°C.

The antisera to these various tissues and blood components, the absorbed antisera, normal rabbit serum and normal rabbit gamma globulin at a concentration of 10 mg/ml in 0.15 M saline were injected intradermally in both lateral thoracic regions of female Holtzman rats weighing 175-225 g using 0.05 ml for each injection. The lesions and sites of injection were examined 2, 4, 6, to 8 hours after injection and then daily except Sunday up to 120 days. There were at least 6 animals in each group injected with one type of serum.

The gamma globulins of normal rabbit and rat sera and of the sheep antisera developed against them were prepared by methanol precipitation(4). All gamma globulins were checked for purity by paper electrophoresis. The sheep antisera to rabbit and rat gamma globulin, resp. each yielded a precipitin titer of 1:2048. The sheep gamma globulins were conjugated with fluorescein isothiocyanate according to the method of Riggs and co-workers(5). The conjugates were chromatographed through a sephadex 75 Column. This was adequate to do away with non-specific staining. The titer of the conjugates was now reduced in both instances to 1:512. The lesions produced by the antisera were removed at 72 hours, immediately frozen in liquid nitrogen and stored at —70°C. Sections were cut in a cryostat. The unwashed sections were stained in a moist chamber with the conjugate for 20 minutes. They were gently washed in buffered saline, mounted in glycerol and examined with a Leitz U-V microscope provided with a U.G. I ultraviolet filter, dark field condenser and blue absorbing objective filter. The specific uptake of the conjugates was blocked with the gamma globulins of the unconjugated sheep antisera 10 mg/ml of 0.15 M saline. With antiglomerular B.M. serum the sites of injection were examined at intervals during the 120 day period of observation.

The initial and late lesions were studied by light microscopy after staining of the sections with hematoxylin-eosin; hemalum-phloxin-saffron; and for reticulum and elastic tissue.

Results. Initial reactions. The antisera to glomerular B.M. and lens capsule produced comparable monophasic erythematous and hemorrhagic reactions(2). The antisera to cartilage, substantia propria of cornea, tendon and vitreous humor produced comparable biphasic reactions(1). The antisera to the nuclear portion of lens, corneal epitheli-

um, liver cells and rat serum yielded mild largely serous reactions, but more prominent than those produced by normal rabbit serum or normal rabbit gamma globulin. The antiserum to red blood cells gave rise to a considerable serofibrinous reaction with some hemorrhage. The latter could be suppressed by prior absorption of the antiserum with rat platelets (unpublished data). The antisera to platelets produced a characteristic serous and massively hemorrhagic lesion with hemorrhagic satellites well away from the injection site. This lesion differed from that produced by anti-glomerular B.M. serum in that (1) there was no erythema and no accompanying specific vasculitis, (2) the lesion could be prevented by prior absorption of the anti-platelet serum with rat platelets, whereas the lesion produced by anti-glomerular B.M. serum was unaffected by absorption of this antiserum with platelets (unpublished data).

Late reactions. The antisera to vitreous humor, nuclear portion of lens, corneal epithelium, liver cells, rat serum, rat red blood cells and rat platelets as well as normal rabbit serum and normal rabbit gamma globulin showed no specific fixation of rabbit gamma globulin to any structures of the skin as tested by immunofluorescence. None of these sera produced late reactions. On the other hand, the antisera to glomerular B.M., lens capsule, cartilage, tendon and the substantia propria of the cornea, all of which showed fixation of rabbit gamma globulin to the epithelial and mesenchymal B.M. of skin by immunofluorescence, exhibited late reactions. However, when the antiserum to glomerular B.M. was absorbed with either myocardium or lung, there was no longer any fixation of rabbit globulin to B.M. The initial reactions were minimal and there were no late reactions. Similarly when the antisera to cartilage, tendon and substantia propria of the cornea were absorbed with lens capsule and glomerular B.M. or with their nonhomologous tissues to the point where there was markedly reduced or no uptake of rabbit globulin by the B.M., there were no late lesions although there still were appreciable initial reactions.

Table I serves to outline these findings and to emphasize the relationship between late reactions and the specific uptake of antibodies by B.M.

Rabbit gamma globulin remained bound to B.M. but with diminished intensity and distribution as judged by immunofluorescence up to 120 days following injection with anti-glomerular B.M. serum. At the same time, there was minimal fixation of rat globulins to B.M. after the fifth or seventh day.

Late reactions generally appeared between 8 and 16 days after injection but were infrequently seen as early as the 5th to the 7th or as late as the 63rd day. These reactions commonly recurred but more than one recurrence was uncommon. In the latter instance the reactions tended to be cyclic at fairly regular intervals. In one such instance, the last recurrence occurred on the 84th day. These late lesions appeared in relationship to the scar resulting from the initial reaction. They often arose at the periphery of the scar as one or more firm infiltrated papules or plateaus with adjacent edema and with or without surface erythema. These lesions might resolve in 24 hours or they might persist for a few days while new ones appeared. Enlargement of the lesions or their confluence resulted at times in a lesion 1.0 cm or more in diameter, involving scar and adjacent tissues and characterized by its elevated, finely nodular, firm infiltrated quality. The reddened surface was frequently ulcerated. Such large lesions took a week or more to resolve.

Microscopically the earliest stage of the late lesion was characterized by vascular dilatation with mononuclear cellular infiltrates about capillaries and venules. With progression, the infiltrate spread and became compact. It was made up chiefly of monocytic-histiocytic cells while lymphocytes and occasional plasma cells occupied the periphery, particularly in relation to newly dilated venules and about arterioles. Other than some peripheral serous exudate, there was little evidence during the evolution of the lesion of acute inflammatory changes. Polymorphonuclear leukocytes were rarely seen and only in conjunction with the fragmentation of

TABLE I. Relationship Between Immunofluorescence of Epithelial and Mesenchymal Basement Membranes of Rat Skin and Late Reactions Following Intradermal Injection of Rabbit Anti-Rat Tissue Sera.

Antiserum to	Tissue used for absorption of antiserum	Immuno-fluorescence	Late reaction
Corneal epithelium	0	0	0
Substantia propria of cornea	0	+	+
Idem	Cartilage	Markedly reduced	0
"	Tendon	" "	0
"	Lens capsule & glomerular BM	0	0
Nuclear portion of lens	0	0	0
Lens capsule	0	+	+
Vitreous humor	0	0	0
Tendon	0	+	+
"	Cartilage	Markedly reduced	0
"	Cornea	" "	0
"	Lens capsule & glomerular BM	0	0
Cartilage	0	+	+
"	Cornea	0	0
"	Tendon	Markedly reduced	0
"	Lens capsule & glomerular BM	" "	0
Glomerular BM	0	+	+
"	Myocardium	0	0
"	Lung	0	0
Liver cells	0	0	0
Rat serum	0	0	0
Rat red blood cells	0	0	0
Rat platelets	0	0	0

monocytic-histiocytic cells or of destroyed muscle fibers.

The lesions were more common in the subcutis and the adjacent muscular panniculus. The dense infiltrate surrounded and destroyed fat cells in the former and striated fibers in the muscular panniculus (Fig. 2). Occasionally the infiltrates occurred principally in the corium particularly in relation to the hair appendages and the arrector muscles. In this location the monocytes were apt to be large and epithelioid in character.

In all instances the cellular infiltrates seemed to be most closely associated with blood vessels (Fig. 1). Venules often showed subendothelial, mural and radiate or concentric rings of perivascular infiltration accounting in part for the nodular appearance of the infiltrate. Comparable infiltrates occurred about small arteries and arterioles with occasional invasion of the media. It was difficult, however, to dissociate the relationship of the infiltrate to the epithelial and mesenchymal B.M. from that to the small blood vessels in close apposition to them.

With resolution the number of mononuclear cells decreased as the number of elongated basophilic fibroblastic cells increased. There were wide lymphatics stuffed with monocytic cells. In brief time, a cellular fibrillar scar replaced the infiltrate while some of the destroyed muscle fibers showed regeneration. In late stages, the scar became less cellular and blended with the one resulting from the initial lesion.

Discussion. Tsuji and coworkers(6) who first reported the exudative hemorrhagic skin lesions in rabbits following intradermal injection of duck anti-rabbit organ sera also described infiltrative, erythematous, hemorrhagic and necrotic lesions appearing at the same sites 5 to 10 days after injection. These lesions were shown to be made up of a dense infiltrate of wandering adventitial and fibroblastic-like cells chiefly in perivascular areas. Such late lesions were not observed in rats injected with rabbit anti-rat organ sera.

The present observations establish that late lesions do occur in the rat and that they have the qualities of a delayed type of hypersensitivity reaction. There can be little doubt on the basis of immunofluorescence and cross absorption of the antisera, that these late reactions are related to the fixation of antibodies to the epithelial and mesenchymal B.M. of skin and to the persistence *in situ*

of such antibodies. While the antibodies to the "common" and "nephrotoxic" antigens in vascular B.M. lead, on initial union, to an acute vasculitis with hemorrhage and those against the "collagenous" antigens presumably play a subordinate role at this stage, it would appear that the fixation of specific antibodies to any or all of these 3 types of antigen in vascular B.M. are of significance in the evolution of the late lesions. Furthermore, specific antibodies fixed to the "common" and "collagenous" antigens of non-vascular B.M. while playing little role in the initial reactions may be significant in these late reactions.

It would be reasonable to assume that these late reactions were due to antibody formed by the host to the injected heterologous antiserum and which has combined with the heterologous antibodies fixed in skin. While such mechanism is implicated in the glomerulonephritis produced by heterologous anti-organ sera injected intravenously(7,8,9), the reaction in skin instead of being Arthus-like, is rather of the delayed hypersensitivity type. This may be attributed to (1) a limited or slow cumulative fixation of the specific rat gamma globulin to the fixed heterologous globulins in skin as compared with freer access of such autologous antibodies to the renal glomerulus, (2) cell-bound antibodies associated with sensitization of the host via

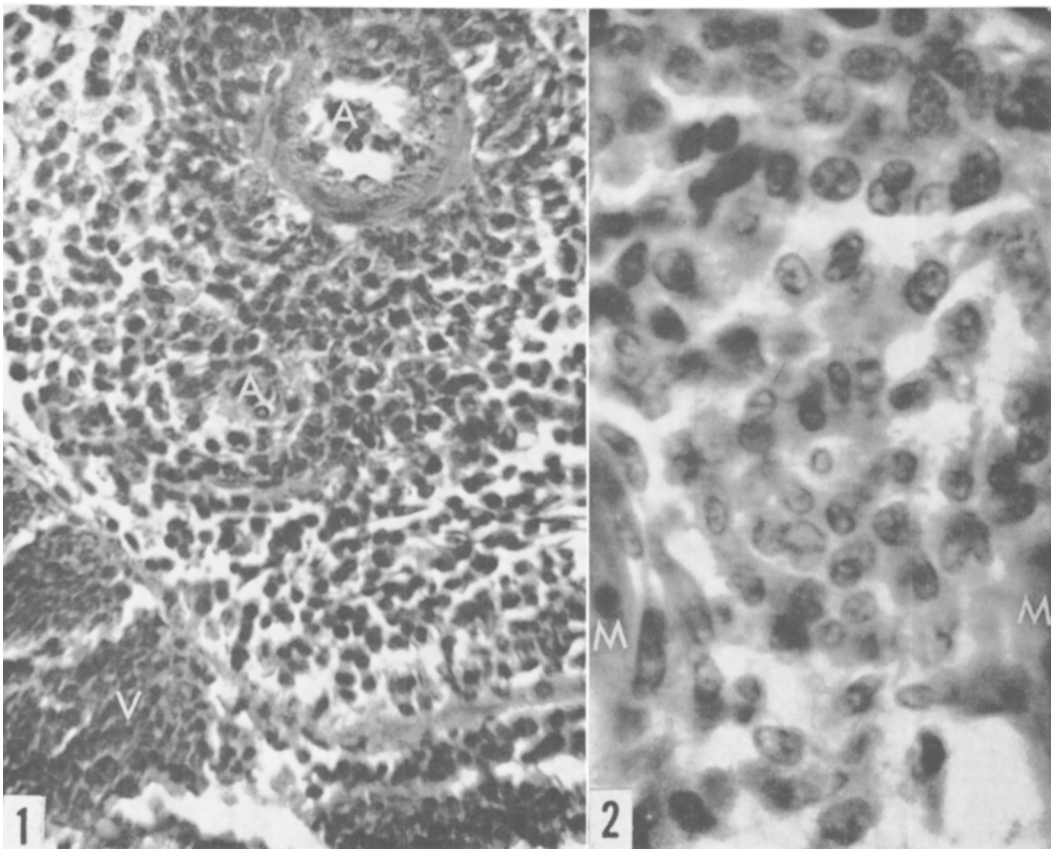

FIG. 1. Concentric rings of monocytic infiltration about arterioles (A) and venule (V). Note medial infiltration of larger arteriole, and subendothelial infiltration of the venule. This was from a suspicious lesion 8 days following injection of anti-lens capsular serum. Hemalum-phloxin-saffron, × 380.

FIG. 2. Monocytic infiltration seen at higher power between muscle fibers (M). This was part of a more extensive lesion 10 days following injection of anti-glomerular B.M. serum. A 0.2 cm nodule appeared at the periphery of the scar on day 9. This was resolving by day 10 but now a new adjoining 0.3 cm lesion had appeared. Hemalum-phloxin-saffron, × 750.

the dermal rather than the intravenous route. Both these mechanisms may be operative in the development of the late lesions.

Summary. Late lesions with the qualities of a delayed hypersensitivity reaction occur in the skin of rats injected intradermally with rabbit anti-rat tissue sera. These lesions are related to the fixation and persistence of antibodies directed against antigens in the epithelial and mesenchymal basement membranes of skin.

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Effect of Brain Removal in Dogs Previously Subjected to Pituitary Stalk Section.* (29233)

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The output of 17-hydroxycorticoids is elevated in dogs after removal of the brain(1-3). The elevation is due to increased ACTH secretion from the isolated pituitary(2,3), but the cause of this increase is unknown. Brain removal entails disruption of the hypophyseal portal vessels, the principal pathway by which blood reaches the anterior pituitary, and the resultant ischemia might cause a prolonged elevation in rate of ACTH secretion. The posterior lobe remnants left in the pituitary fossa might also play a role because the corticotropin-releasing factor known to be present in this tissue(4) might reach the anterior lobe via the vascular anastomoses between the 2 lobes(5). To explore these possibilities further, we have studied 17-hydroxycorticoid output in the adrenal vein before and after brain removal in dogs in which the pituitary stalk had been sectioned and a plastic plate inserted between the hypothalamus and the pituitary 2 to 10 weeks previously. An abstract reporting the results of some of these experiments has been published(6).

Methods. The pituitary stalk was sectioned via the transtemporal approach(7) in male mongrel dogs anesthetized with pentobarbital. After temporal craniectomy and opening of the dura, the temporal lobe was elevated, the oculomotor nerve was cut, and the hypophyseal stalk was visualized and sectioned with a blunt instrument. A 2-component epoxy polyamide resin (resiweld 620, H. B. Fuller Co.) was mixed and loaded into a small disposable plastic syringe attached to a blunt No. 17 gauge needle. As the temporal lobe and the hypothalamus were gently elevated with a flat-blade retractor, the needle was placed between the hypothalamus and hypophysis, the plunger firmly pressed, and the point of the needle gradually moved about. The freshly mixed resin was viscous, but flowed sufficiently well before hardening to form a solid cap molded over the superior surface of the hypophysis. We have found this method of interposing a barrier between the hypothalamus and the pituitary to be very satisfactory.

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