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Study with Fluorescent Antibody of Fate of Intradermally Injected Proteins in Rabbits.* (20232)

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The fate of intravenously injected antigenic substances has been followed with interest because of its bearing on the mechanism of destruction of foreign materials in relation to the stimulation of antibody formation, and the distribution of the lesions of experimental serum disease obtained with different antigens(1-4). The present report presents data concerning the sequence of events following intradermal injection of two antigenic proteins in normal and passively sensitized rabbits and in rabbits sensitized with homologous antigen plus Freund's adjuvants.

Methods. 2.5-3.0 kg rabbits were used throughout. Antiserum pools, containing 1.21 and 0.50 mg N/ml of antibody against Armour's crystalline ovalbumin (Ea) or Armour's bovine Fraction II (BGG), were obtained from animals injected with these antigens in combination with Freund's adjuvants. Some rabbits were passively sensitized the day before the test by intravenous injection of 4.0 mg of antibody N(5). Others were sensitized 3 weeks earlier by inoculating the two front foot pads with 0.05 ml of an emulsion containing 20 mg protein antigen and 3 mg heat-killed tubercle bacilli per ml of mineral oil (Bayol F). Test injections of 2 or 10 mg of 3 times recrystallized Ea(6) or BGG in 0.10 ml of physiological saline were made intradermally on the shaved outer aspect of the hind leg above the heel. Animals were sacrificed 1, 6, 24, and 48 hours after the test injection. The injected area of skin and the homolateral popliteal lymph node, and sometimes other tissues, were removed and quick frozen. 4-6 μ cold-box sections of these tissues were stained with fluorescein-labelled, homologous antibody, obtained from the serum pools described above, and examined under the fluorescence microscope. The technic of labelling antibody with fluorescein and the staining methods are described by Coons *et al.*(7-9). Controls demonstrating the specificity of the fluorescent staining included: absence of staining by heterologous labelled antibody, and inhibition of specific staining in sections treated first with unlabelled antibody. Sections stained by the Giemsa technic, were also examined.

Results. The normal rabbits showed minimal microscopic changes. Those sensitized passively or with the aid of adjuvants presented typical Arthus reactions, 10 to 25 mm in diameter, maximal at 6 or 24 hours, and remaining conspicuous at 48 hours in the adjuvant group. The microscopic picture included edema and polymorphonuclear exudation, marked at 1 hour in the deeper skin layers, involving all layers at 6 and 24 hours, and massive around vessels and hair follicles. Deep areas of hemorrhage were seen, particularly in the responses to Ea. At 48 hours, there was an increase in fixed cells and other mononuclears. The adjuvant sensitized rabbits showed more intense and prolonged reactions, including marked fixed cell activation in the papillary layer, some infiltration of lymphoid elements at 24 and 48 hours, and necrosis,

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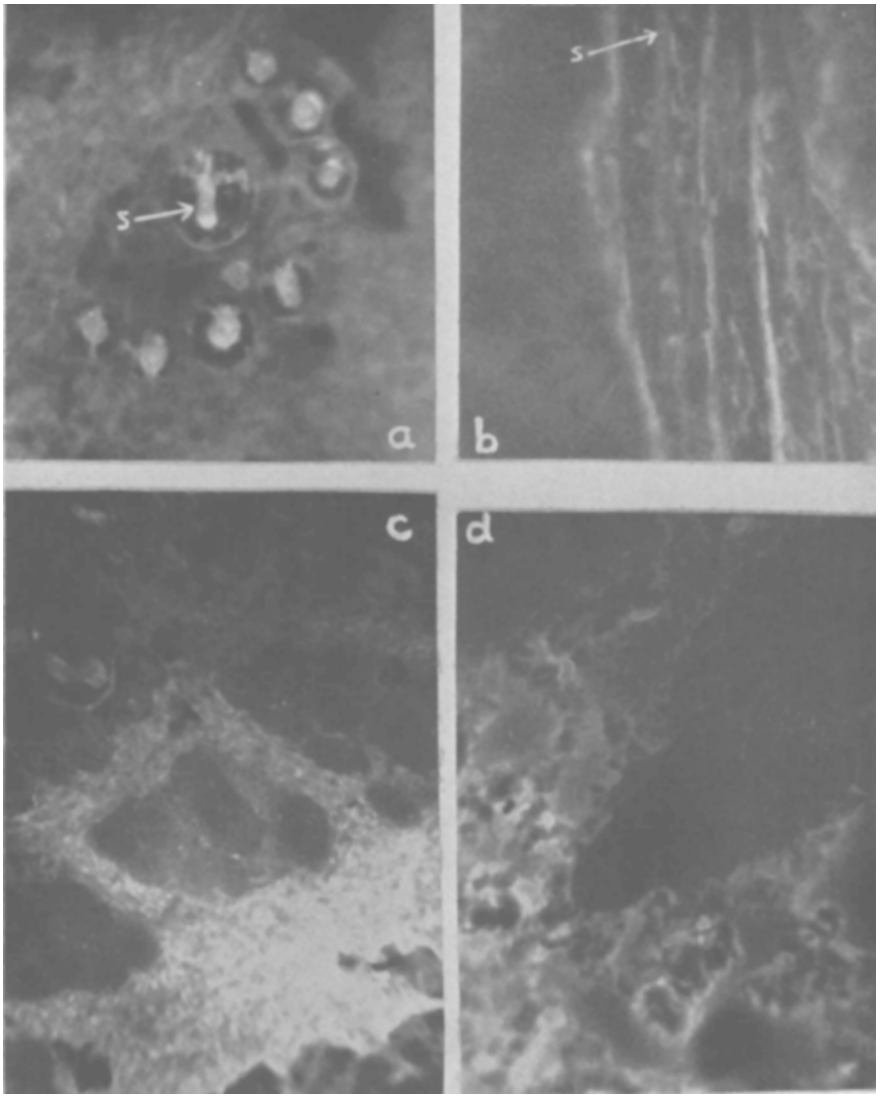

FIG. 1. One hr skin specimens, stained with fluorescent antibody, × 150. a. Ea, 2 mg, in normal rabbit. Antigen around and between hair follicle cells. Hair shafts (s) have own reddish fluorescence but no antigen. b. Gamma globulin, 2 mg, in normal rabbit. Hair follicles cut longitudinally. c. Gamma globulin, 2 mg, in passively sensitized rabbit. Antigen in deep connective tissue and around hair follicles. No antigen penetration into deep follicles themselves. d. Gamma globulin, 10 mg, in adjuvant-sensitized rabbit. Edematous connective tissue near deep follicles showing lakes of extracellular antigen.

especially of polymorphonuclears. The lymph node changes consisted of edema, hemorrhage, and polymorphonuclear infiltration involving the medullary and cortical sinuses and the nodules and parallel in degree to the changes in the skin. In the Freund sensitized rabbits, the reaction was prolonged and included extensive polymorphonuclear necrosis and loss of cortical tissue at 24 and 48 hours.

The amount of antigen present in the injected skin site and in the draining lymph node at various intervals after the test injection was evaluated according to both the intensity of staining with fluorescent antibody and the extent of antigen distribution. In the normal, Ea was almost entirely gone from the skin site at 24 hours and was transiently visible in the draining node at 6 hours. In the

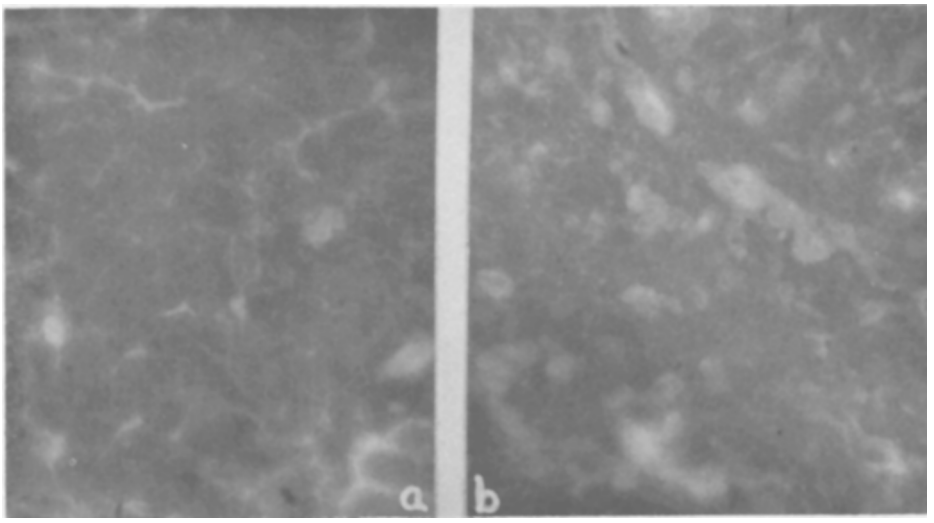

FIG. 2. Twenty-four hr skin specimens stained with fluorescent antibody, × 300. Both are Ea, 10 mg, in adjuvant-sensitized rabbits. a. Deep connective tissue, showing antigen between collagen bundles and contained in several histiocytic elements. b. Connective tissue immediately beneath epidermis, showing numerous antigen-containing macrophages.

passively sensitized animal, Ea disappeared from the skin as quickly as in the normal but was visible in the node both earlier (1 hour) and later (24 hours), as though the sensitizing antibody slowed its escape. In the adjuvant sensitized rabbits, this slowing effect was seen in both the skin site and the node, where more intense staining was also observed at all times. BGG, presumably because of its greater molecular size, disappeared slowly from the skin, where it was still found at 48 hours in normal as well as sensitized rabbits. It also disappeared slowly from the nodes and often gave more intense staining than the comparable Ea preparation. The fixation effect noted in Ea sensitized animals was also apparent in both skin and nodes of the rabbits sensitized to BGG.

At 1 and 6 hours, the antigen was spread widely in the deeper skin layers, while more superficially it was limited to the area immediately above the injection site. It was seen characteristically in large extracellular pools in the edematous deeper tissues and along the outside of hair follicles. It formed clearly defined borders along the collagenous connective tissue fibers as though adsorbed, and penetrated the hair follicles at an intermediate level to surround the hair shaft. The picture was identical for the 2 protein antigens studied, and is illustrated in Fig. 1. In the normal and passively sensitized rabbits, only a few cells of the histiocytic type, in the deep connective tissue, contained antigen in their cytoplasm at one and 6 hours. As antigen disappeared, however, from the follicles and the superficial skin layers first, small amounts could be demonstrated in macrophages (in cytoplasm and nuclei) near the skin surface. Antigen was not identified in the infiltrating polymorphonuclears; nor was there evidence of increased phagocytosis in the passively sensitized group. The last identifiable antigen to remain was usually seen in the deep connective tissue, and was still largely extracellular. In the adjuvant-sensitized animals, more phagocytosis was apparent in all parts of the tissue (Fig. 2), almost entirely by histiocytic elements containing antigen in nuclei as well as cytoplasm (some of these cells may have been fibroblasts). Antigen containing macrophages in the papillary layer were very conspicuous in this group, particularly at 6 and 24 hours. Here too the antigen was visible longest in the deep connective tissue, mostly in extracellular pools. None was seen at any time in the epidermis.

In the popliteal nodes of all groups of animals, antigen was found first in the peripheral and medullary sinuses and was extracellular.

Often a sinus bordering one lymphoid nodule contained a considerable amount of antigen while another quite close contained none. Later, much of the antigen was contained in reticular cells (in cytoplasm and nuclei) in the peripheral sinuses; and similar cells or small groups of cells were seen within the nodules. At the same time, considerable numbers of lymphoid cells at the periphery of the nodule showed antigen in their cytoplasm. No antigen was seen in the germinal centers. The sequence of events in passively immunized rabbits was the same as in normals. The whole process was speeded up and intensified in the adjuvant sensitized animals, most of the visible antigen being intracellular at 6 hours, while it remained largely in extracellular lakes in the normals. Such antigen as remained at 48 hours was entirely contained in histiocytic cells scattered throughout medulla and cortex.

Discussion. Protein injected into the rabbit skin behaves like protein injected by other routes in the mouse or rabbit. Coons and his collaborators(1) have shown that intravenously injected material rapidly appears in the connective tissue, where it is adsorbed on connective tissue fibers and taken up by reticulo-endothelial cells and possibly fibroblasts. It is not seen in epithelial tissue except in certain specialized organs, but is transiently present in lymphoid cells of spleen and lymph nodes. It disappears from the tissues at a rate apparently determined by its molecular size, and remains in reticulo-endothelial cells and connective tissue after disappearing from other sites. Thus, no characteristic distribution could be found to distinguish the intradermal from other routes of administration.

It was an unexpected finding of the present study that polymorphonuclear cells, which participated actively in the skin responses, did not contain antigen recognizable by the fluorescent antibody technic. Either these cells did not take up antigen or they destroyed it immediately after ingestion. In the normal rabbit ear chamber, dye-protein is taken up largely by macrophages, beginning at about 4 hours(10). In our skin sections, phagocytosis of antigen by macrophages was seen at one hour but was more definite at 6 hours, even in the adjuvant sensitized group.

It was surprising that the presence of homologous antibody in the passively sensitized rabbits did not bring about more obvious changes in the sequence of events. The great excess of antigen may have obscured any actual differences. In the passively sensitized group and in those animals sensitized with protein plus adjuvants, antigen was recognizable earlier and later at the local site and in the draining node. This finding suggests that diffusion of antigen into the general circulation was retarded, whether by the local edema or by combination with antibody. Otherwise it would appear inconsistent with the increased rate of antigen breakdown occurring in actively and passively immunized animals (11). The increased phagocytosis of antigen seen in the adjuvant group may correspond to the increased phagocytic capacity of histiocytic cells seen in the tuberculin type of sensitivity(12). The delayed, necrotic character of the skin reactions in this group of animals suggested that they may have depended in part on the tuberculin type of sensitivity.

Summary. 1. In the rabbit, intradermally injected crystalline egg albumin and bovine gamma globulin are taken up by histiocytic cells both locally and in the peripheral and medullary sinuses of the draining lymph node. Considerable quantities remain extracellular and presumably diffuse away to be dealt with elsewhere in the body. Polymorphonuclear cells, though present in considerable numbers, are not found to contain intact antigen. 2. In the draining node many lymphoid cells containing antigen are seen in the peripheral portions of the lymphoid nodules. 3. Egg albumin disappears much more quickly than bovine gamma globulin from the injected skin site and the draining node. 4. Passive sensitization and to a greater extent sensitization with Freund's adjuvants result in a slowing of both antigens' disappearance from skin and node. Adjuvant sensitized animals also show speeding up and intensification of phagocytosis of antigen in both localities.

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Clotting of Citrated Plasma by Bacteria which Destroy the Anticoagulant: Effect of Sodium Fluoroacetate. (20233)

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When certain bacteria are incubated in citrated plasma, they frequently remove the anticoagulant, citrate, and the blood clots (1,2). Clotting is a dependable end point of utilization of citrate and could offer a simple means of studying bacterial metabolism of citrate. The present study was conducted to clarify how the rate of bacterial utilization of citrate in plasma is related to bacterial growth and to clotting. In addition, sodium monofluoroacetate and potassium cyanide, substances known to affect metabolism of citrate in experimental animals, were tested for their action on the clotting of citrated plasma by bacteria.

Method. In all experiments, organisms were tested for clotting by inoculating 1.0 ml of citrated human plasma with 0.1 ml of a culture grown for 24 hours in tryptose phosphate broth. Inoculated plasmas were incubated at 37°C and examined daily to 8 days for clotting. Clotted plasmas were completely jelled. Unclotted plasmas remained entirely fluid. One ml of ACD solution (1.32 g sodium citrate, 0.48 g citric acid, and 1.47 g dextrose per 100 ml) was used for 4 ml of blood. Results were recorded only if heavy bacterial growth had occurred. Quantitative determinations of citrate were made on whole clotted plasmas, including fluid within the clot, by the method of Lardy(3). The *rate of clotting* of citrated plasma containing increasing concentrations of sodium monofluoroacetate or potassium cyanide was determined after inoculation with various strains of *Aerobacter aerogenes* or enterococci. The rate at which *bacteria destroyed citrate* was determined in mixtures containing 3 parts of citrated plasma

TABLE I. Clotting by Bacterial Consumption of Citrate. Results obtained by growth of 116 strains in citrated blood.

Bacteria	Strains tested, No.	Strains which clotted citrated human blood, No.
A. aerogenes	18	16
A. cloacae	2	2
K. pneumoniae	8	6
Salmonella species	5	4
Diphtheroid	2	2
Ps. aeruginosa	15	7
B. alcaligenes	1	1
Paracolon species	4	2
E. freundii	8	3
E. coli	20	12
Shigella species	2	1
Staphylococcus coagulase—	15	2
Enterococcus	13	4
Str. viridans	3	0
Totals	116	62