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Extracutaneous Delayed Hypersensitivity, Particularly in the Guinea-Pig Bladder*

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Summary. In outbred guinea-pigs with a low level of hypersensitivity to hen egg albumin (HEA) at a time when circulating antibody was not detectable, unaggregated HEA in extracutaneous tissues was rapidly lost from the injection site and did not elicit lesions of delayed hypersensitivity. Aggregated HEA, however, was retained at the depot site and produced lesions in the bladders of similarly prepared guinea-pigs. In Hartley guinea-pigs with a high level of hypersensitivity, as measured by skin reactions, unaggregated HEA, though rapidly dissipated from depot sites, elicited lesions in the bladder. Aggregated HEA, though retained at injection sites, did not produced reactions of delayed hypersensitivity in kidney, testis and muscle. The inflammation of delayed hypersensitivity did not influence the disappearance rate of labelled HEA from the lesion, while the inflammation of an Arthus response was associated with retention of labelled antigen at the injection site.

INTRODUCTION

The lesion of cutaneous delayed hypersensitivity is characterized by the accumulation of mononuclear cells around venules and nerves of the connective tissue in which specific antigen has been deposited. Such focal pathology represents a systemic immune response, as was clearly demonstrated by the passive transfer of delayed hypersensitivity with lymphoid cells from immunized guinea-pigs (Chase, 1945). In organs other than the skin it should be possible, therefore, to produce a pathological process of this type of immunity and a number of investigators have elicited lesions in many extracutaneous sites in animals with hypersensitivity to mycobacteria or their protein derivatives (Rich, 1944). The morbid process in these extracutaneous tissues, however, has been complicated by the use of complex antigens, such as micro-organisms, by the presence of classical circulating antibody, and by tissue destruction. Lesions in extracutaneous tissues have not been produced in immunized animals by a single purified protein in the absence of detectable circulating antibody.

In this study, two different but interdependent facets of extracutaneous delayed hypersensitivity were studied in guinea-pigs, in which there was no detectable circulating antibody. First, the suitability of several organs and tissues to provide soil for the development of the lesion was examined. The bladder of immunized guinea-pigs was found to be a good organ in which to elicit a specific delayed hypersensitivity reaction.

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Second, the disappearance rates of labelled antigen from the different organs and tissues were determined in animals with delayed hypersensitivity, and in others with circulating antibody. These rates of disappearance were influenced by the type of immune response and by the form of antigen used, i.e. aggregated or non-aggregated.

MATERIALS AND METHODS

Animals

White and albino guinea-pigs (non-Hartley or 'outbred'), weighing 350–400 g, were obtained from local sources, and Hartley strain guinea-pigs of similar weight were purchased from Camm Research Institute, Inc., Wayne, New Jersey.

Antigens

The antigens used were: five times recrystallized hen egg albumin (HEA, K and K Laboratories, Inc., Jamaica, New York); bovine γ-globulin (BGG, Armour Pharmaceutical Co., Kankakee, Illinois); and rabbit γ-globulin (RGG, Cohn Fr. II from Pentex, Inc., Kankakee, Illinois).

HEA was iodinated (^{131}I from Iso/Serve, Cambridge 39, Massachusetts) according to the method of Weigle and Dixon (1959), except that the iodide was added to the HEA in a 0·05 M PO_4 buffer at pH 11. The [^{131}I]HEA produced was not detectably denatured by this procedure; it reacted with anti-HEA antibody in agar to produce a single precipitin band and also elicited specific delayed hypersensitivity lesions. The specific activity of the [^{131}I]HEA was usually 1 μc ^{131}I per 5 μg HEA protein, with less than one iodine atom per HEA protein molecule.

To prevent the rapid loss of antigen from the injection site, HEA was aggregated by using bis-diazotized benzidine (BDB) to form HEA–BDB–HEA complexes, as done by Ishizaka and Ishizaka (1960). Stock BDB was prepared as described by Gordon, Rose and Sehon (1958), frozen at −78° in 1 ml aliquots and stored at −30°. For aggregation of HEA, stock BDB was diluted 1:15 in a 0·15 M isotonic phosphate buffer and added to the HEA diluted in the same buffer so that the final protein concentration was 100 μg/ml. This mixture was kept at room temperature with occasional agitation for 10 minutes and then 2 mg of L-tyrosine was added to react with the remaining uncoupled BDB. Control HEA unaggregated antigens were treated identically except that no BDB was added. The amount of BDB added to the HEA was adjusted to give a 3:1 molecular ratio. That BDB was capable of binding proteins into complexes was demonstrated by coupling [^{131}I]HEA to BGG and by showing that BGG–anti-BGG precipitin lines in Ouchterlony plates also contained isotope by autoradiography. The persistence of HEA antigenicity after BDB aggregation was demonstrated by specific fluorescence with an anti-HEA fluorescein conjugate in skin sections taken 24 hours after skin testing with the aggregated HEA. When the anti-HEA fluorescein conjugate was adsorbed with HEA, no staining was found in skin lesions elicited by aggregated HEA.

Immunization

Guinea-pigs were immunized by injection into the four foot-pads of 5 μg of protein emulsified in 0·1 ml of complete Freund's adjuvant (containing 2 mg tuberculin). Tests

for delayed hypersensitivity were made 5 days later, at a time when delayed hypersensitivity was present with no demonstrable circulating antibody by Preer technique, or by PCA, as was also shown by Coe and Salvin (1964).

Antigen testing

Skin tests were performed by injecting the flank skin intradermally and marking the bleb with a felt marking pen. Under ether anaesthesia the urinary bladder was protruded into the inferior angle of a lower abdominal incision and gently held by the fingers. Using a 0·25 ml glass syringe with a 30 gauge needle, the serosa was pierced at the dome of the bladder and the needle carefully advanced under the serosa for several millimetres. The appearance of a subserosal bleb upon delivery of the protein indicated a successful injection. After some practice, the injection of protein into this site could be consistently duplicated. The kidney injections were subcapsular, also accomplished by advancing the needle several millimetres under the capsule before effecting delivery. A small haemorrhagic spot marked the injection site. Testing antigen was deposited directly into the testis. Muscle injections were marked by co-ordinate placement of sutures. The quantity of both non-aggregated and aggregated HEA used for eliciting reactions was 5 μg in 0·05 ml saline.

Radioactive determinations

Promptly after deposition of the isotopically labelled protein in the organs, the amount of initial radioactivity was determined by counting the animal in a whole body counter and was calculated as the 100 per cent level. Following a suitable interval, the injection site or whole organ was removed and counted for residual radioactivity. For skin test determinations, a circular piece of skin, 35 mm in diameter, was removed. For bladder determinations, the bladder neck was exposed and amputated. After slight enlargement of the urethral orifice, the bladder was thoroughly rinsed with 15 ml saline introduced inside through the urethral orifice and the whole organ was counted. For determining radioactivity in the kidney the whole organ was removed and counted. Other organs were not counted. Final calculations were expressed as a percentage of the dose delivered, corrected for decay, background, etc.

Histologic examination

Tissues were fixed in buffered 10 per cent formalin and three to four haematoxylin and eosin sections of each tissue or organ were studied. A bladder lesion was considered positive when there was an accumulation of mononuclear cells in the test site 18–24 hours after delivery of antigen with less than 10 per cent polymorphonuclear leucocytes among the infiltrating cells. In the skin a positive lesion consisted of at least three distinct clusters of mononuclear cells around vessels and/or nerves in any one section, scattered mononuclear elements in the dermis, and less than 5 per cent of polymorphonuclear leucocytes in the infiltrate.

RESULTS

EXTRACUTANEOUS DELAYED HYPERSENSITIVITY

Bladder

When non-aggregated HEA was used as eliciting antigen in six outbred guinea-pigs which had been sensitized 5 days earlier with HEA, only one exhibited a positive reaction

in the bladder at 24 hours (Table 1, column 3). Six control guinea-pigs, immunized with either RGG or BGG, displayed no positive reactions in the bladder, and five guinea-pigs, which were immunized with HEA and had circulating antibody, all showed Arthus reactions in the bladder wall.

For comparative purposes both the experimental and control animals were simultaneously skin tested with non-aggregated HEA. In the experimental group of six, skin lesions were minimal (Table 1, column 5). One guinea-pig with a gross lesion of 23×22 mm also developed a lesion in its bladder. In the control group prepared with a heterologous protein, skin test sites were negative grossly and microscopically. In the group immunized with HEA and also showing circulating antibody, the typical Arthus response was found in the skin.

When aggregated HEA was used as eliciting antigen, ten of eleven outbred guinea-pigs displayed positive reactions in their bladders and none of five controls, immunized with BGG or RGG, showed a positive bladder reaction (Table 1, column 3).

Since these outbred guinea-pigs exhibited evidence of a low degree of hypersensitivity, as noted by the relatively poor skin test responses, Hartley strain guinea-pigs were used.

When non-aggregated HEA was injected into the bladders of Hartley guinea-pigs, all of ten animals showed positive reactions (Table 1, column 6), and none out of seven controls prepared with BGG or RGG exhibited a positive response in the bladder wall. In the former group, simultaneous skin tests revealed good gross responses in most of the guinea-pigs (Table 1, column 8), and characteristic, moderately cellular, microscopic lesions. In the latter control group, skin test sites were negative grossly and microscopically.

When aggregated HEA was used, there were five out of five positive reactions in the bladder and none out of four was positive in the controls. It was not possible to distinguish grossly or histologically any differences in bladder wall response between those animals receiving unaggregated HEA and those injected with aggregated HEA.

The histology of the inflammatory response to specific antigen in the bladder wall was characterized by masses of mononuclear cells, predominantly lymphocytes, filling the mucosal connective tissues of the dome (Figs. 1 and 2). Blood vessels were dilated and congested. A pattern of mononuclear cells clustered around vessels and nerves, so characteristic of the skin lesion of delayed hypersensitivity, was not apparent, possibly because the infiltrate was so dense. This clustering pattern was occasionally seen, however, in the connective tissue septa between the bladder muscle bundles and in the subserosa, particularly when the response was severe and extensive (Fig. 3). A few polymorphonuclear leucocytes were always visible in the mucosal masses of cells, but these were dispersed as single elements and represented less than a few per cent of all the infiltrating cells. The lesion was fairly sharply demarcated and tissues beyond the zone of inflammation did not appear altered in any way. What was most striking was the relatively large number of infiltrating cells, as compared with the number found in skin

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FIG. 1. A lesion of delayed hypersensitivity in the bladder of a guinea-pig immunized with 5 μg of HEA and tested 5 days later with 5 μg of HEA. The site was removed 24 hours after delivery of the specific antigen. Large numbers of mononuclear cells, mostly lymphocytes, occupy the connective tissues beneath the bladder epithelium (Ep). $\times 75$.

FIG. 2. A higher magnification of the same lesion reveals the predominant cell type to be chiefly lymphocytes. Arrows point to polymorphonuclear leucocytes. $\times 300$.

FIG. 3. A cluster of mononuclear cells around a bladder vessel at the edge of a delayed hypersensitivity lesion. This pattern of infiltration was not frequently seen in the bladder, but was characteristically present in skin lesions. V, vein; M, muscle; $\times 145$.

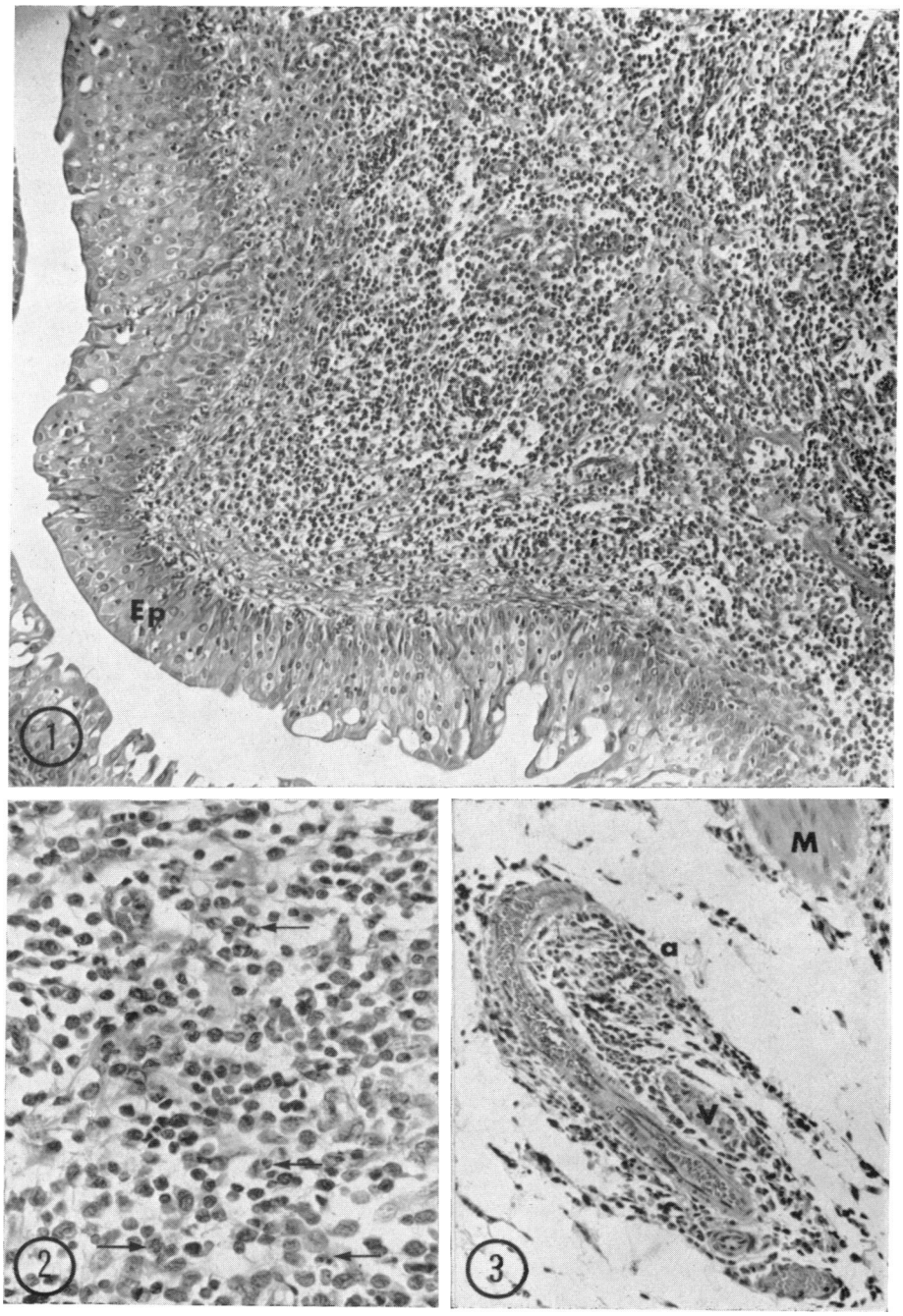

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TABLE 1

RESPONSE OF BLADDER AND SKIN TO HEA AND AGGREGATED HEA IN HYPERSENSITIVE AND CONTROL GUINEA-PIGS

Immunization	Testing antigen	Outbred			Hartley		
		Bladder		Skin	Bladder		Skin
		Positive reactions/ No. tested	Antigen retained—24 hours (per cent of dose)	Area of induration (mm)	Positive reactions/ No. tested	Antigen retained—24 hours (per cent of dose)	Area of induration (mm)
HEA	HEA	1*/6	0·13†, 0·13, 0·1, 0·1, 0·07	23×22†, 15×10, 10×10, 5×5 (2), 0×0	10*/10	0·2, 0·2, 0·1, <0·1, <0·1	25×25 (2), 20×20, 18×18, 18×16, 16×16, 16×15, 12×12, 10×10, 0×0
BGG or RGG	HEA	0/6	0·16, 0·13, 0·13, 0·13	Negative	0/7	0·2, 0·2, 0·2, 0·1×(4)	Negative
HEA	Aggregated HEA	10*/11	33, 28, 28, 26, 25, 25, 18		5*/5	32, 33	
BGG or RGG	Aggregated HEA	0/5	27, 27, 11, 16, 24		0/4	33, 30	
HEA with circulating antibody	HEA	5‡/5	3, 2·5	Arthus			

* Mononuclear infiltration. † Positive bladder reaction. ‡ Arthus reaction in bladder.

sites tested at the same time as the bladder. Three injected bladders of hypersensitive Hartley animals were removed 8 hours after testing with specific antigen and no lesions were found by microscopic examination.

Kidney, testis, muscle

Aggregated HEA was injected into eight kidneys, three testes, and three muscles in guinea-pigs with delayed hypersensitivity to HEA. Histologic examination of the 24-hour lesion did not reveal any instance of mononuclear infiltrate in the kidneys and testes. One test site in muscle showed a few mononuclear clusters in a perivascular distribution in the interstitial spaces.

DISAPPEARANCE RATES OF LABELLED ANTIGEN

Bladder

Non-aggregated [^{131}I]HEA rapidly disappeared from the injection site of the bladder in both the outbred and Hartley guinea-pigs and, indeed, the curves describing the rates of loss were quite similar (Figs. 4 and 5). At 8–9 hours after deposit of the antigen, approximately 1 per cent of the dose remained, and at 24 hours only 0·1–0·2 per cent was counted (Figs. 4 and 5, Table 1, columns 4 and 7). Furthermore, there was no

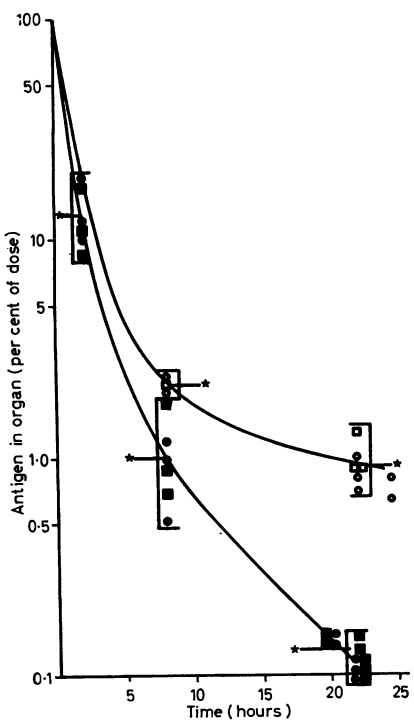

FIG. 4

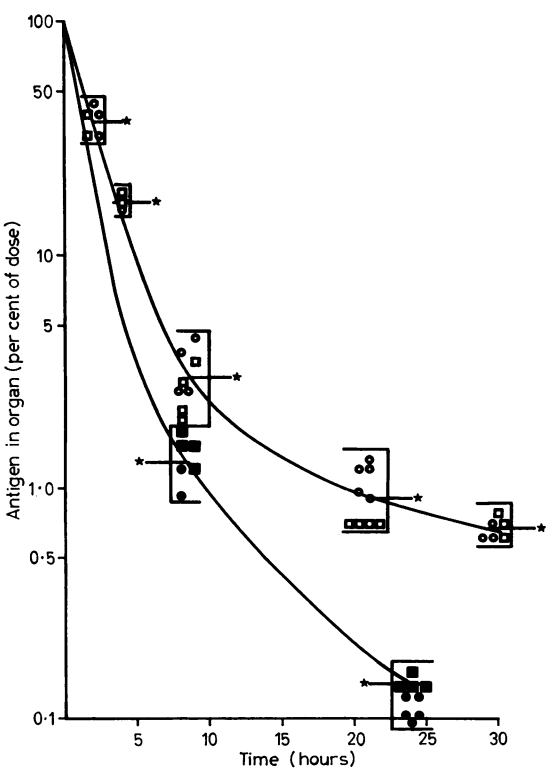

FIG. 5

FIGS. 4 and 5. Semilog plots of the disappearance of radioactivity from bladders and skin sites of outbred guinea-pigs (Fig. 4) and Hartley guinea-pigs (Fig. 5) after injection of [^{131}I]HEA. Bladder: ●, hypersensitivity present; ■, control. Skin: ○, hypersensitivity present; □, control. ★, Mean of observations at one time.

difference of rate of loss in guinea-pigs which had been sensitized with HEA and manifested delayed hypersensitivity reactions and those which had been immunized with a heterologous protein, such as BGG or RGG, and developed no lesion. However, in the presence of circulating antibody and of a local Arthus reaction, retention of specific antigen in the bladder was significantly greater at 9 hours (mean of 16 per cent) and at 24 hours (mean of 2·7 per cent), about fifteen times greater than in animals without circulating antibody.

Aggregation of [^{131}I]HEA with BDB permitted much larger amounts of antigen to be retained in the bladder, ranging from 11 to 33 per cent of the dose at 20–24 hours after deposit (Table 1, columns 4 and 7). Again, no significant differences in antigen retention were noted between animals which had delayed hypersensitivity and those immunized with a heterologous protein, or between the outbred and Hartley groups.

Since it was difficult to quantitate the degree of hypersensitivity either by gross or microscopic measurements of the bladder lesion, it was not possible to relate the amount of antigen retained to the severity of a lesion. This was particularly true for the Hartley guinea-pigs which responded vigorously to the test antigen, either non-aggregated or aggregated, in the bladder and skin. In the outbred guinea-pigs, however, in which the level of delayed hypersensitivity was presumed to be low, as manifested by gross skin reactions, non-aggregated HEA produced a positive bladder reaction in but one of six tests, while aggregated HEA produced positive bladder lesions in ten out of eleven tests.

Skin

Disappearance rates of [^{131}I]HEA from the skin were charted and found to be significantly slower than those observed in bladder (Figs. 4 and 5). At 8–9 hours, about 3 per cent of injected antigen was counted at the test site (about 1·0 per cent in bladder at this time); at 20–30 hours from 0·6 to 1·4 per cent of the antigen was retained at the skin test site compared with 0·1–0·2 per cent in bladder. The slower loss of labelled HEA from skin depots might explain why positive responses were obtained in the skin while there were no lesions in five of six bladders of outbred animals. When aggregated [^{131}I]HEA was used 14 and 16 per cent of the dose was retained at 24 hours in the skins of two immunized and two control guinea-pigs, respectively.

Kidney

Although no lesions were elicited in kidneys of immunized guinea-pigs by a test dose of aggregated HEA, it was desirable to learn whether the aggregated [^{131}I]HEA was retained in kidney. In the kidneys of five outbred animals, the retention of aggregated antigen at 24 hours ranged from 4 to 18 per cent of the dose (average 12 per cent) and there was no pathology by microscopic examination.

Aggregated [^{131}I]HEA was not used in testis and muscle, since histologic study of test sites injected with aggregated HEA had previously shown no lesions.

DISCUSSION

It was possible to elicit lesions of delayed hypersensitivity in an extracutaneous site using the laboratory model described and employing a soluble antigen. The guinea-pig bladder proved to be an appropriate organ for the development of this type of pathology,

indeed, the only organ of those tested, while kidney, testis and muscle did not provide a soil for lesions to evolve. At the time of testing, 5 days after initiation of immunization with HEA, there was no detectable circulating antibody, nor evidence of an Arthus response in the microscopic lesions. Although polymorphonuclear leucocytes were always present, they comprised less than 10 per cent of the infiltrating elements and were dispersed sparsely in the mass of mononuclear cells. Blood vessels were dilated and congested with erythrocytes, but their walls were unaltered. (The small vessels within an Arthus reaction exhibit a smudging and necrosis of their walls.) Characteristic of the bladder pathology, in distinction to the typical skin reaction, were the sheets of lymphoid cells within the expanded mucosa, and the paucity of nodular aggregates surrounding vessels and nerves. There was no valid way to compare in any quantitative sense, either macro- or microscopically, the pathology in the bladder with the lesion of delayed hypersensitivity in the skin. Nevertheless, the number of infiltrating cells was obviously greater in the bladder connective tissue than in the dermis when the lesions of the two organs were removed and examined from the same immunized guinea-pig or from animals of a group treated at the same time with the same reagents.

The absence of pathology in extracutaneous organs other than bladder after testing for delayed hypersensitivity was not readily explained. In the model used here, unknown anatomical or physiological factors inherent in the kidney, testis and muscle possibly prevented the appearance of lesions after injection of test antigen. Certainly in many microbial diseases, such as, among others, tuberculosis and histoplasmosis, any tissue may harbour the focal pathology characteristic of hypersensitivity, but the conditions of the disease process are quite different from those of this laboratory model. In active infection with micro-organisms, the antigen is usually replicating, tends to remain in the affected locus, calls forth circulating antibodies and causes tissue destruction. Similarly, in autoimmune disease perpetrated in the laboratory animal, the antigens are fixed tissue antigens, there are often circulating antibodies to complicate the inflammatory process, and cellular destruction and breakdown products obscure the evolution of the pathology.

Two factors did influence the development of delayed hypersensitivity lesions in the bladder and skin and seemed to be reciprocally related. These were the level of hypersensitivity in the host and the retention of antigen in the organ. In outbred guinea-pigs, the degree of hypersensitivity that could be generated in this model was presumably low, as expressed by the minimal gross and histological skin lesions present 24 hours after testing. When non-aggregated HEA was injected into the bladders of such guinea-pigs only one of six developed a lesion. The disappearance rate of labelled antigen was rapid, only 1 per cent of the dose being found at 8 hours and less than 0·15 per cent at 24 hours after testing. At this latter time, a mean of about 0·8 per cent of the dose was counted in the skin test site, approximately eight times more than in the bladder. As shown in Fig. 4, the rate of disappearance of [^{131}I]HEA from the skin injection site was slower than the rate of loss from the bladder. In part, this might have explained why skin lesions were obtained while the bladder reactions were absent in five of six tested outbred animals. By contrast, when aggregated HEA was injected into the bladders of prepared outbred guinea-pigs, ten of eleven developed a florid lesion and the amount of [^{131}I]HEA retained in the organ at 24 hours was 26 per cent of the test dose. These observations suggested, then, that when the degree of hypersensitivity was marginal, retention of antigen at the injected site played an essential role in the appearance of a

lesion. The retention of antigen might have had a two-fold beneficial effect on the production of a lesion. Not only was more antigen present at the test site to react with immune cells, but also peripheral desensitization by the dissipated antigen presumably was less.

In Hartley guinea-pigs, the level of hypersensitivity that could be generated was presumably high, as manifested by the size of 24-hour skin lesions and by the histological infiltrate. When non-aggregated HEA was injected into the bladders of these immunized animals, ten of ten developed strong mononuclear reactions. The disappearance rate of labelled test antigen from the bladder was rapid, about 1 per cent of the dose being present at 8 hours and less than 0·15 per cent at 24 hours after testing. A perusal of Figs. 4 and 5 reveals that the rates of disappearance of [^{131}I]HEA from the bladders and skins of Hartley guinea-pigs were quite similar to those of the outbred animals. These observations suggested, then, that when the degree of sensitivity was high, retention of antigen in the injected site played a minor role.

We have used the phrase 'level of hypersensitivity' to indicate quantitative differences between outbred and Hartley guinea-pigs in their gross and histological reactions to a test dose of specific antigen. Unfortunately, at present, there is no real quantitative measure of this immune state and the meaning of the phrase in terms of cells, cell-bound antibodies, or transfer factors is obscure. There are, nonetheless, visible degrees of response.

A final comment is worth emphasis. The disappearance rates of [^{131}I]HEA from bladders and skin injection sites were almost the same whether the host guinea-pig had a focal lesion of delayed hypersensitivity or not. This type of immune state did not affect the retention of test antigen. By contrast, in guinea-pigs in which antibody to HEA was demonstrable in the circulation and in which Arthus reactions developed, [^{131}I]HEA was retained at injection sites. In bladders at 9 hours after delivery of test antigen, approximately 16 per cent was present and at 24 hours about 3 per cent. These quantities were fifteen and twenty times greater, respectively, than those in bladders of guinea-pigs with delayed hypersensitivity. Opie (1923) and Korngold, Stahly, Dodd and Myers (1953) have also demonstrated retention of antigen in Arthus reactions, and Korngold *et al.* (1953) and Menkin (1930) have reported that inflammation *per se* was a barrier to the loss of antigen from its depot. Rich and McKee (1934), however, have demonstrated that antigens may be held at the injection site in the absence of inflammation, provided specific antibodies were present to fix the antigens. Previous studies by Long (1959) and Oort and Turk (1963) on antigen disappearance in delayed hypersensitivity lesions showed some instances of antigen retention, although small amounts of circulating antibody were possibly responsible for this. In the model presented here, it was apparent that the inflammation of delayed hypersensitivity did not retard escape of protein antigen.

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