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Immune elimination and immune retention: the relationship between antigen retained in the foot and the elicitation of footpad swelling

J. G. TEW, T. E. MANDEL* & PATRICIA L. RICE *Department of Microbiology, Medical College of Virginia, Richmond, Virginia 23298, U.S.A. and *The Walter and Eliza Hall Institute of Medical Research, Parkville 3050, Australia*

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Summary. We determined whether (1) long term antigen retention is present in distal sites after footpad challenge of immune mice; (2) if antigen retained in the foot is selectively localized; and (3) if the foot is adversely affected by the retained antigen. Mice immune or non-immune to human serum albumin (HSA) were injected in the hind footpads with ^{125}I-HSA. In immune mice rapid clearance of radiolabel occurred in the liver, lungs, kidney, blood and urine but radiolabel was retained in the hind feet, draining lymph nodes and spleen. Non-immune mice rapidly cleared radiolabel from these sites. Autoradiography revealed that most of the radiolabel in the feet was in flexor tendons and tendon sheaths. Electron microscope autoradiography indicated that antigen was associated with collagen at the tendon surface, but not with cells or cell processes. Radiolabel solubilized from the feet, lymph nodes and spleen could be specifically precipitated with rabbit anti-HSA. Histological examination of the tendon and surrounding tissues did not show that retained antigen was causing inflammation or chronic tissue damage. Nanogram levels of antigen could elicit swelling in the sensitized foot even if the antigen was injected at a remote site, but mice immunized by other routes or against other antigens did not show footpad swelling. Antigen retained on collagenous tissues may induce hypersensitivity and thus play a role in rheumatic diseases.

Correspondence: Dr T. E. Mandel, The Walter and Eliza Hall Institute of Medical Research, Post Office, Royal Melbourne Hospital, Victoria 3050, Australia.

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INTRODUCTION

The immunological consequences of maintaining small quantities of immunologically active antigen in immunized animals for long periods of time are not clear. In previous studies, we reported evidence supporting the concept that antigen persisting in lymph nodes operates in conjunction with specific antibody in a feedback system which maintains and regulates serum antibody levels (Tew, Self, Harold & Stavitsky, 1973; Tew & Stavitsky, 1974; Greene, Tew & Stavitsky, 1975; Tew & Mandel, 1978; Tew & Mandel, 1979). It has also been proposed that antigen persisting in joints provides a chronic immunological stimulus which could lead to some forms of arthritis (Cooke, Hurd, Ziff & Jasin, 1972; Jasin & Cooke, 1978). It also appears that long term persistence of complex polysaccharides, such as pneumococcal polysaccharide, maintains a state of specific tolerance or paralysis (Dixon, Maurer & Weigle, 1955; Siskind, Patterson & Thomas, 1963). In addition to reports indicating antigen retention in lymph nodes and joints, we recently noted the retention of antigen in the feet of footpad immunized

mice (Tew & Mandel, 1979). The long-term nature of this phenomenon was indicated by the half-life of approximately 6 weeks (Tew & Mandel, 1979). Analysis of the retention mechanism in the feet indicated that although it was antibody-dependent, it did not require the complement system or intact antibody since cobra venom factor decomplemented mice or mice passively immunized with $F(ab')_2$ fragments of antibody retained antigen as well as or better than controls (Tew, Mandel & Miller, 1979b). The possibility that the large pool of retained antigen in the foot served as a depot to maintain antigen in the nodes was also discounted since amputation of the foot had no effect on long term antigen retention in the draining nodes (Tew & Mandel, 1979).

The objectives of the present study were to determine in which tissues immune retention occurs in relation to immune elimination in footpad-challenged mice. We further sought to establish where anatomically the antigen was located in the foot, and finally, to determine if the foot was adversely affected by the retained antigen. The data indicate that immune retention was most striking in the draining lymph nodes and the feet. Autoradiography indicated that retained antigen was preferentially localized on the flexor tendons and the tendon sheaths. Retained antigen did not appear to damage the tendon during the long maintenance period, but it rendered the foot specifically hypersensitive to antigen and small amounts of additional antigen, even if injected at a distal site, would elicit footpad swelling.

MATERIALS AND METHODS

Antigen

Human serum albumin (HSA) was used either in native form or heat-aggregated when used for priming. To follow the antigen *in vivo*, HSA was radioiodinated by the chloramine-T method (Greenwood, Hunter & Glover, 1963). The specific activity of the radiolabelled antigen was between 18 and 25 μCi/μg.

Animals

CBA/H WEHI mice of both sexes, obtained from the specific pathogen free colony at the Walter and Eliza Hall Institute, or CBA/J male mice from Jackson Laboratories, Bar Harbor, ME, were used throughout this study. Animals aged 6–8 weeks were primed, boosted, and used within the next 7 months. Animals injected with ^{125}I-HSA were given water containing KI (50 mg/l) 2 days before injection and maintained on this water thereafter to minimize uptake of radioactivity in the thyroid.

Immunization

Most mice were primed by injecting both hind footpads with 0·5 mg heat-aggregated HSA emulsified in Freund's complete adjuvant in a 50 μl volume. However, for certain experiments, mice were injected intraperitoneally or subcutaneously in the loose skin of the neck. Two to four weeks later, all mice were boosted in the same sites with the same amount of antigen.

Processing of tissues

Three to twelve weeks after boosting, mice were injected in both hind footpads with 2·5 μg ^{125}I-HSA in phosphate-buffered saline (PBS). At specific times thereafter the mice were killed by cervical dislocation and various tissues were carefully removed and weighed (to 0·1 mg) and radioactivity was counted in a Packard Gamma Counter. The tissues studied were the draining lymph nodes (popliteal and inguinal), spleen, liver, lung, kidney, the foot (distal to the ankle joint) and the hind limb between the knee and hip joints. The skin from the leg as well as skin from the neck, a site well removed from the area of antigen administration, and a measured volume (20 μl) of blood and bladder urine were also studied. After weighing (to 0·1 mg), the foot was further dissected into four anatomically distinct components (long flexor tendons, long extensor tendons, soft tissue of the sole, and plantar skin). The foot tissues were individually weighed and their radioactivity was counted as above. The radioactivity in the tissues and fluids was converted to picograms of antigen and the results were expressed as antigen retained per mg of sample. This allowed a direct comparison to be made in terms of specific activity per unit mass of tissue.

In some instances, tissues were fixed in 10% formalin or Bouin's solution and processed for light microscope autoradiography. Five-micron sections were cut, dipped in Kodak NTB2 emulsion and exposed for periods ranging from 2 to 56 days. The sections were developed with Dektol and stained with haematoxylin and eosin.

Electron microscope autoradiography

Tissues were fixed in a mixture of paraformaldehyde (2%) and glutaraldehyde (2·5%) in 0·08 M cacodylate buffer. After rinsing overnight in cacodylate buffer and post-fixation in 2% OsO_4 followed by 2% aqueous

uranyl acetate, the tissues were dehydrated in acetone and embedded in Spurr's low viscosity resin. Thin sections (pale gold interference colours) were placed on collodion-coated glass slides. These were then coated with a 20 nm carbon layer and dipped in Kodak NTE emulsion. The slides were stored in the dark at 4° in a dry atmosphere for 4–6 weeks before development in Dektol. The developed sections were stripped off the slides, placed on unsupported 200 mesh copper grids and examined without further staining in a Philips 300 electron microscope operating at 40 kv.

Solubilization of the ^{125}I-labelled antigen from lymph nodes

Eleven days after injecting ^{125}I-HSA into immunized mice, the tissues were removed, cut into small pieces and teased apart with stainless steel wire screens in 10 ml of PBS containing 5 M guanidine hydrochloride and 100 μg per ml of non-radioactive HSA (Tew, Mandel & Burgess, 1979a). This mixture was incubated at 37° in a water bath for 30 min and then dialysed against several changes of PBS at 4°. The dialysate was centrifuged at 800 ***g*** to remove particulate material. Eighty to ninety per cent of the radiolabel originally present in the lymph node and spleen could be solubilized by this procedure and was present in the supernatant fluid. However, we were able to solubilize only 20–50% of the radiolabel in most other tissues and the skin was especially difficult. In most tissues other than lymphoid tissue, aggregates formed which were difficult to keep suspended.

Co-precipitation

Supernatant fluid from the dialysed guanidine hydrochloride treated tissue, representing approximately 150 μg of HSA (about 1·5 ml), was taken and sufficient rabbit anti-HSA was added to establish equivalence and precipitate the antigen. The mixture was allowed to stand overnight at 4° and the precipitate was collected by centrifugation at 800 ***g***. The supernatant fluid was discarded and the sample was resuspended in 1 ml of PBS. The sample was centrifuged as described above, washed a second time, and the radioactivity retained in the precipitate was counted.

Footpad swelling

One to seven months after the final immunization, HSA immune and non-immune CBA/J mice were challenged with HSA or egg albumin (EA) in saline. Prior to challenge, duplicate measurements of the lateral aspect of each footpad were obtained using a Starrett dial micrometer and the results for each foot recorded to the nearest 0·01 mm. Additional measurements were obtained in the same manner at various times post-challenge to monitor the course of swelling. At each time point, the amount of swelling for each foot was determined. The mean and standard error of the mean for each group of mice (four to six animals per group) was calculated and the results expressed in footpad swelling units. One footpad swelling unit is equal to 0·1 mm thickness.

RESULTS

Immune and non-immune CBA mice were injected in both hind footpads with ^{125}I-labelled HSA. The radiolabel served as a marker to compare the patterns of antigen elimination in the two groups. The results were expressed as pg antigen per mg tissue to provide a standard for comparison. The clearance of the radiolabel from the liver, lung, kidney, blood and urine of immune and non-immune mice are shown in Fig. 1a. The radiolabel in these tissues and fluids was cleared rapidly in immune animals and the results illustrate the well established immune elimination phenomenon. However, when the elimination pattern obtained in the feet was compared in the same two groups of mice, a very different result emerged (Fig. 1b). For the first 3 days, antigen elimination by the immune and non-immune animals was similar but by the sixth day the non-immune animals had eliminated much more antigen than the immune mice. Statistical analysis of the data using Student's *t* test indicated that the immune animals retained significantly more antigen than the non-immune animals from day 6 onwards ($P < 0{\cdot}01$ for days 6, 11 and 28). Thus, in contrast to immune elimination, an immune retention phenomenon appeared in the foot. The foot was dissected and samples were taken to determine whether antigen was selectively localized to particular tissues. The long flexor tendons near the site of injection were the major site of antigen localization, but the more distal extensor tendons, separated from the injection site by the metatarsal bones, also retained antigen. Autoradiographs of transverse and longitudinal sections of the whole footpad at times immediately after challenge with ^{125}I-HSA showed that the injection site containing the majority of the silver grains was in the subcutaneous tissues and did not involve deeper tissues including the overlying flexor tendons. By contrast after some days the injection site was cleared of

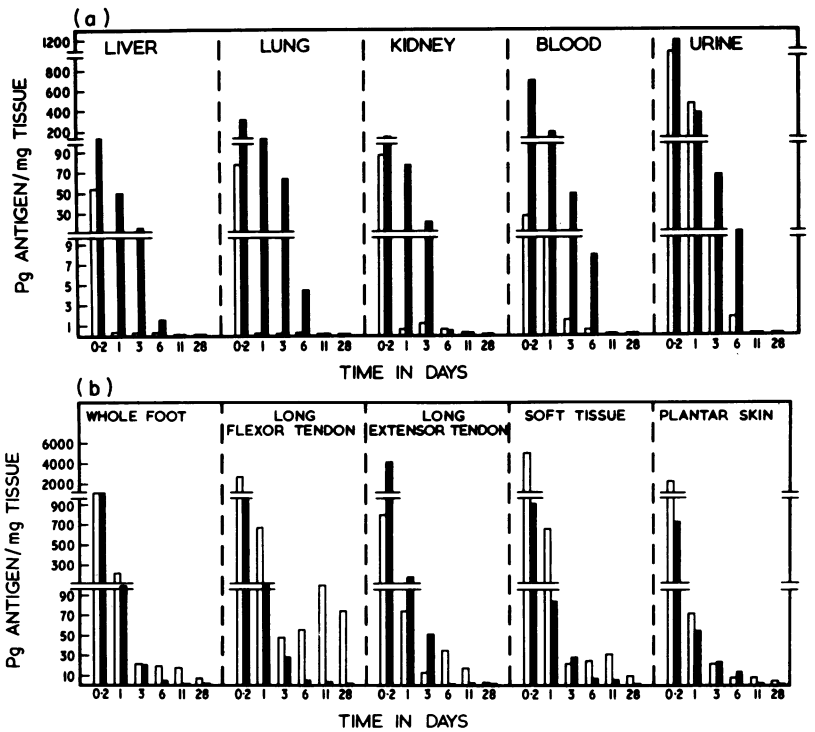

Figure 1. The kinetics of antigen clearance from various tissues of immune and non-immune mice. The amounts of antigen retained, standardized as picograms of HSA per milligram of tissue are represented by the open bars for immune and closed bars for non-immune animals. Figure 1a shows the fate of antigen over a period of 28 days after challenge in non-lymphoid organs. Figure 1b represents the fate of antigen in the whole foot and in the various tissue components of the foot over 28 days. These animals were injected in each hind footpad with 2·4 μg of ^{125}I-HSA (23 μCi/μg) and at various times groups of three mice were killed and the various tissues and fluids collected, weighed and counted. A small sample of the radiolabelled HSA was counted and used as a standard to convert the counts per minute into picograms of antigen.

radioactivity but grains were present around the tendons. The variability recorded for tendons in Fig. 1b may be related to which part of the tendon and how much of the tendon was sampled and autoradiographs showed labelling was uneven along the length of the tendons. Some retained antigen was also associated with the small flexor foot muscles after removing the major tendons. Antigen was also selectively associated with the plantar skin, although the amount was variable and generally relatively low (4·0 $\pm$ 0·9 pg/mg plantar skin in immune mice *v.* 1·1 $\pm$ 0·6 pg/mg in non-immune mice at day 28).

Immune retention was also seen in the lymph nodes and spleen (Fig. 2a). The amount of antigen retained per mg of tissue was similar to that seen in the foot and the differences between immune and non-immune mice were also statistically highly significant. In addition, antigen was found in the leg as well as in the skin from the leg and neck (Fig. 2b). However, the amount per mg of tissue was only one-tenth to one-twentieth as much as in the draining lymph nodes or in the feet.

Autoradiographs of the foot indicated a striking localization of radiolabel around the tendons and the tendon sheath (Figs 3 and 4). In comparison, the muscle and bones remained free of label. Occasionally, longer exposure revealed the presence of some antigen associated with cells in the epidermis. Electron microscopic autoradiography of the tendons indicated that the retained antigen in this location was not cell associated. Rather, antigen appeared to be bound to sites on the exposed collagen running between cells (Fig. 5).

In previous work, 5 M guanidine hydrochloride was used to solubilize antigen retained in the draining lymph nodes and in joints (Cooke *et al.*, 1972; Tew &

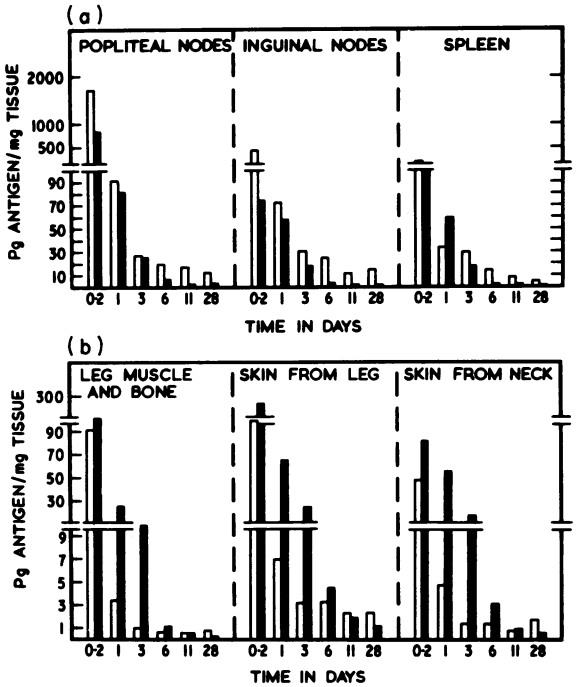

Figure 2. Antigen removal from various tissues of immune (open bars) and non-immune (filled bars) mice over a 28 day period. Figure 2a shows the selective retention of antigen in the lymphoid organs of the immune mice. Figure 2B shows low levels of retention in the skin from the leg and slight retention in back skin and leg muscles and bone in immune mice. These tissues were from the same animals used to prepare the data in Fig. 1.

Mandel, 1979). Similar experiments were performed with tissues examined in this study. The level of radiolabel in the intact organs or large tissue samples 11 days after antigen injection is shown in Table 1. By this time, the rapid immune elimination phase is over and the radiolabel in the tissues is relatively stable. Co-precipitation results obtained on radiolabel released from these tissues are presented in Table 2. Most of the radiolabel released from the kidney, liver, skin and lung was not specifically co-precipitable whereas most of the radiolabel retained in the foot, the hind legs, and the lymphoid organs was co-precipitable. The sites in the animal where immune retention occurred correlated with the ability to specifically co-precipitate the solubilized radioactivity.

It did not appear from histological examination of the tendons and surrounding tissues that retained antigen was producing chronic tissue damage. However, the foot appeared to be hypersensitive. Nanogram amounts of antigen injected into feet of mice retaining antigen from the initial immunization elicited swelling (Fig. 6). Even minute amounts of antigen could elicit swelling in the primed foot but

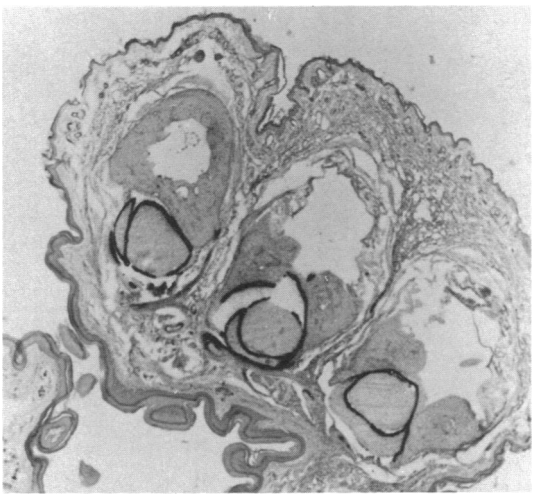

Figure 3. Transverse sections of the footpad showing antigen localization around the long flexor tendons. This animal was challenged with ^{125}I-HSA 6 days before being killed. Note the heavy labelling of the flexor tendons and the virtual absence of label elsewhere (× 34, 2 day exposure).

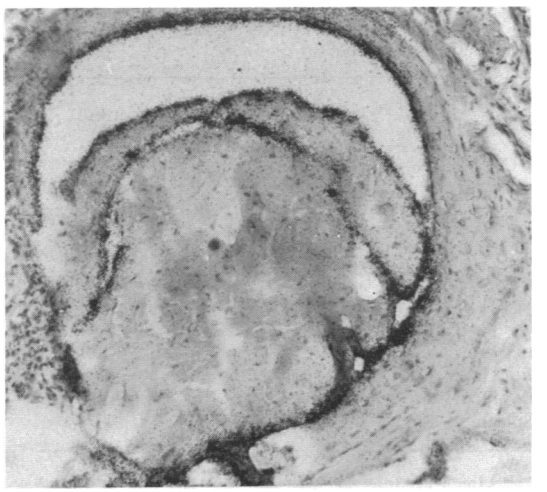

Figure 4. Antigen localization to the surface of a long flexor tendon and to the lining of the tendon sheath. Most of the silver grains are located at surface structures and show little penetration into the substance of the tendon (× 210, 29 day exposure).

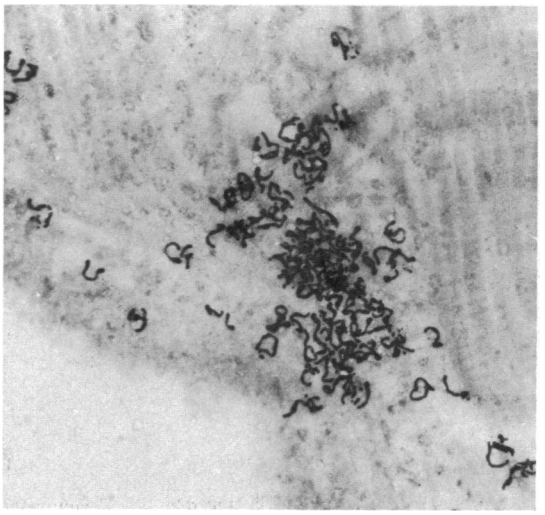

Figure 5. Electron microscope autoradiograph of a long flexor tendon showing the dense accumulation of silver grains at its surface (× 50,000, exposed for 4 weeks).

much larger amounts also elicited some swelling in the contralateral foot. In a typical experiment mice were immunized by injecting antigen into only one hind footpad and were later challenged by injecting antigen in both hind footpads. In the experiment illustrated in Fig. 6, a 7 month interval between immunization and challenge was allowed to elapse indicating the long-term nature of the phenomenon. In this experiment, different groups of mice were injected with 10 ng, 100 ng and 1 μg of HSA. Footpad thickness was measured 7 and 26 h after challenge. At the 10 and 100 ng doses, the previously injected foot, which contains persisting antigen, swelled, and this was apparent already at 7 h post-challenge. In contrast, these low doses either failed to induce swelling or swelling was minimal in the unprimed foot. At the higher 1 μg dose both feet swelled indicating that the unprimed foot would swell when challenged with a sufficiently large dose of antigen. More remarkably, administering antigen at sites remote from the foot, i.e. injecting antigen into the peritoneal cavity or introducing antigen into the stomach by gavage, produced footpad swelling (Fig. 7). The swelling was obvious within 1 h after administering HSA, was maximal by 6 h, and the feet remained swollen for over 48 h. However, footpad swelling only occurred in animals that had antigen retained in the foot from the initial immunization 6 weeks earlier. The feet of normal animals or immune animals primed by injecting antigen by other routes did not swell. Furthermore, footpad swelling was antigen-specific.

Table 1. The localization of radiolabel in HSA-immunized and non-immunized mice eleven days after injecting ^{125}I-HSA*

Tissue site	Radioactivity retained/mouse (c.p.m. ± SE)	
	Immune mice	Non-immune mice
Kidney†	2,960 ± 280	5,170 ± 1,100
Liver	7,250 ± 800	30,800 ± 10,400
Back skin	12,000 ± 2,400	13,200 ± 1,850
Leg skin	34,850 ± 6,000	24,000 ± 7,200
Lung	760 ± 160	13,200 ± 1,600
Leg muscle	14,100 ± 5,400	7,400 ± 1,360
Foot‡	118,000 ± 17,100	9,000 ± 1,560
Popliteal node	6,530 ± 2,800	168 ± 28
Spleen	19,100 ± 8,200	520 ± 80

* Mice were injected with 2 μg ^{125}I-HSA in both hind footpads.

† The c.p.m./tissue was for the whole organ except for skin where standardization was on the basis of a 300 mg skin sample.

‡ The foot was cut from the leg at the ankle joint.

Table 2. The specific coprecipitation of radioactive material released from various tissues by treatment with 5 M guanidine hydrochloride*

Tissue or organ	Radioactivity coprecipitated (%)	
	HSA-anti-HSA	EA-anti-EA
Kidney	2	1
Liver	5	0
Back skin	25	9†
Leg skin	27	26†
Lung	35	4
Leg muscle	60	1
Foot	71	3
Popliteal lymph node	73	3
Spleen	80	1
HSA standard	90	5

* The tissues were taken from three immune animals and pooled prior to treatment with guanidine hydrochloride.

† The skin became very viscous after treatment with guanidine hydrochloride. These high non-specific values were attributable to the problems encountered in washing this material.

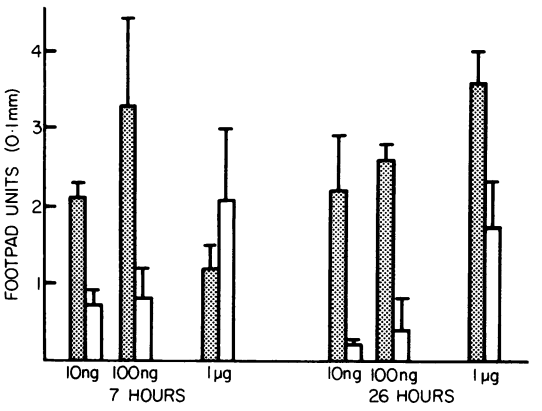

Figure 6. Footpad swelling elicited with nanogram levels of antigen in feet retaining antigen. Mice were primed and boosted with HSA in one hind footpad and were challenged 7 months later by injecting HSA in both hind footpads. Different groups of mice received 10 ng, 100 ng and 1 μg of HSA in 50 μl saline. Footpad swelling was measured at 7 and 26 h post-challenge. The filled bars represent the response in feet used for priming and open bars represent the unprimed contralateral feet.

Administering a non-specific antigen, i.e. egg albumin, into footpad-primed animals did not induce inflammation (Fig. 7).

Histological examination of the swollen feet at 24 or 48 h revealed numerous mononuclear cells in the tissues. However, all primed animals had been injected with antigen in Freund's complete adjuvant and a large number of mononuclear cells remained in the footpad. This background of inflammatory cells made it difficult to show clear histological differences between the antigen-primed feet before and after challenge.

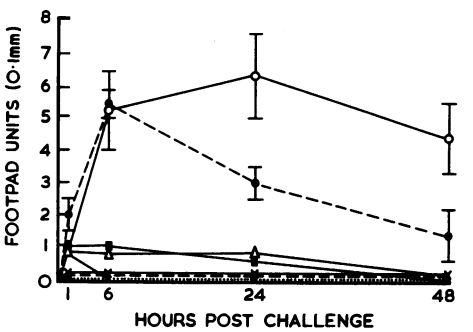

Figure 7. The specific induction of footpad swelling by an antigen administered either by intraperitoneal injection or into the stomach by gavage. Eight groups of animals were studied, representing various combinations of route of immunization and site of challenge: (○) footpad-immunized, challenged with 100 μg HSA in stomach by gavage; (●) footpad-immunized, challenged with 10μg HSA i.p.; (■) i.p. immunized, challenged with 10 μg HSA i.p.; (△) footpad-immunized, challenged with 10 μg EA i.p.; (· · · ·) footpad-immunized, challenged with 100 μg EA gavage; (×——×) non-immune, challenged with 10 μg HSA i.p.; (×– – –×) footpad-immunized, saline injected i.p.; (▲) Immunized subcutaneously behind neck, challenged with 10 μg HSA i.p.

DISCUSSION

The results reported here indicate that whether immune elimination or immune retention of antigen was observed depended entirely upon which tissues or fluids were examined and on the immune status of the animal. The draining lymphoid organs and the tendons near the site of injection were the primary areas for immune retention and some antigen appeared to be retained in the skin. It is probable that antigen retention on tendons and antigen retention in joints (Cooke *et al.*, 1972) represents expression of the same phenomenon at different sites. However, the ability to retain antigen does not appear to apply to all collagenous tissues. In a series of experiments using immune mice, rats, and rabbits, we were unable to demonstrate immune retention in collagenous tissues around the teeth or the palate when radioactive antigen was injected in these sites (unreported observation). It is possible that the collagenous tissues around the surface of the tendon, the tendon sheath, and the joints are particularly efficient in trapping and retaining immune complexes. If such a special association exists, it could relate to the fact that these are the surfaces where the collagenous tissues are mobile. It is also conceivable that normal movement in a joint or tendon could be inhibited by even limited damage at such a critical site.

Recent experiments in our laboratory indicate that passively administered specific antibody is necessary and sufficient to get antigen retention on tendons of normal mice. The intact complement sequence does not appear to be required since decomplementation by cobra venom factor does not diminish antigen retention (Tew *et al.*, 1979b). *In vitro* work by Hollister & Mannik (1974) indicates that antigen–antibody complexes may be non-specifically trapped in the collagen network. The experiments of Hawkins (1971) and Henson (1971) suggest that collagen layers may present a non-phagocytosable surface which protects the antigen from phagocytic cells and promotes long term antigen retention.

The nature of the cell in skin which retains radiola-

bel is not certain. The location of Langerhans cells in the dermis and epidermis as well as their surface features makes them an attractive possibility. Recent work on Langerhans cells indicates they have Fc receptors and Ia antigens (Rowden, Lewis & Sullivan, 1977; Stingl, Wolff-Schreiner, Pichler, Gschnait & Knapp, 1977). Certainly the Fc receptor would facilitate capture of antigen–antibody complexes in immune animals. The possibility that antigen retained in the skin plays a major role in the elicitation of footpad swelling has not been excluded.

It has been suggested that the chronicity associated with some forms of arthritic disease could be attributed to the persistence of antigen (Cooke *et al.*, 1972; Jasin & Cooke, 1978). However, others question the significance of the persisting immunological stimulus (Fox & Glynn, 1977). Histological examination of the tendons and surrounding tissues of our mice did not indicate that retained antigen was mediating structurally detectable damage, at least for several weeks after challenge. This result would be consistent with the view that the retained immunological stimulus is not directly causing tissue damage. However, our data suggest that retained antigen has rendered the local site hypersensitive and subject to repeated episodes of swelling upon challenge with specific antigen. Our data further suggest the possibility that antigen could arrive at such sites via the circulation from a remote site. For example, microbial products from a respiratory or gastrointestinal infection could be the source of specific antigen and such infections could provide the antigenic stimulus needed to elicit episodes of swelling. If this interpretation is correct, it could explain how episodes of arthritis or tendonitis could occur in the absence of an infection at the inflammed site.

The immunological consequences of immune retention are just beginning to be understood. Considerable evidence is now available indicating that retained antigen plays a major role in maintenance and regulation of serum antibody levels (Graf & Uhr, 1969; Bystryn, Schenkein & Uhr, 1971; Weigle, 1975; Tew & Mandel, 1978). It appears that antigen retained on dendritic cells plays a vital role in initiating immunological memory (Klaus & Humphrey, 1977; Thorbecke, Romano & Lerman, 1974) and it seems likely that the persisting antigen plays a continuing role in the maintenance of memory. Our results also suggest that the retained antigen could play a role in the maintenance of local hypersensitivity which may be important in the pathogenesis of disease states such as arthritis or tendonitis.

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