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Prolonged antigen half-life in the lymphoid follicles of specifically immunized mice

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Summary. The kinetics of clearance of ^{125}I from the popliteal lymph nodes and feet of human serum albumin (HSA)-immunized mice was studied following the injection of [^{125}I]-HSA into the hind footpads. Antigen was cleared from both locations rapidly for the first few days. The antigen half-life ($T_{\frac{1}{2}}$) during this period was only a matter of hours. By the end of the first week, however, the rate of clearance in both sites had changed markedly. The antigen $T_{\frac{1}{2}}$ in the node between the first and sixth week was 8·1 weeks (95% confidence interval between 5·1 and 20 weeks) and the antigen $T_{\frac{1}{2}}$ in the foot was 6·1 weeks (95% confidence interval between 3·7 and 16·6 weeks). There was, however, about twenty times more radioactivity in the feet than in the popliteal nodes. Autoradiography of popliteal lymph nodes revealed that initially antigen was trapped in the medulla, subcapsular sinus, superficial cortex and around lymphoid follicles. During the first few days antigen was cleared from all sites except the follicles. The radioactivity initially trapped in the medulla, subcapsular sinus, and superficial cortex appeared to have been associated with macrophages. Studies with peritoneal macrophages indicated an antigen $T_{\frac{1}{2}}$ in these cells of 2 h (95% confidence interval between 1·5 and 3 h). The initial rapid clearance of antigen trapped and catabolized by macrophages and the long-term retention of antigen in the follicles is probably attributable to trapping and retention by follicular dendritic cells. The large pool of antigen trapped in the foot did not appear to serve as a depot to replace antigen degraded in the node, since amputation of the foot did not alter the level of antigen retained in the node. The long antigen $T_{\frac{1}{2}}$ in the lymph node follicles indicates that antigen is available in the lymph node to play a role in the maintenance and regulation of immune responses for many months or even years.

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INTRODUCTION

The need for antigen in the initiation of immune responses is well known but its continuing role in their maintenance and regulation is less well established. Evidence supporting a continuing role for antigen comes from experiments which show that antibodies of differing specificities are maintained within narrow limits for prolonged periods without the administration of fresh exogenous antigen (Graf & Uhr, 1969; Bystryn, Graf & Uhr, 1970; Kitces, Tew & Greene, 1975). Such long-term regulation can be explained by the cyclical appearance of antibody-producing cells in the lymphoid organs. (Britton & Möller, 1968; Weigle, 1975). The period between such cycles is related to the level of circulating antibody (Britton & Möller, 1968). Antibody production itself appears to be regulated by a feedback mechanism which only permits fresh antibody synthesis when cir-

culating levels of antibody decline (Schenkein, Bystryn & Uhr, 1971).

We recently demonstrated the induction of antibody synthesis *in vitro* in the absence of freshly added antigen (Tew & Mandel, 1978; Tew, Self, Harold & Stavitsky, 1973). This occurred in lymph node fragments or in cell cultures prepared from nodes in the lymphatic drainage path of the original site of antigen injection. By using ^{125}I-labelled antigen we showed that radioactivity was retained only in draining lymph nodes and that those lymph-node fragments which contained the radiolabel produced antibody whereas those fragments which were not radioactive failed to produce antibody (Tew & Mandel, 1978). Furthermore, the amount of 'spontaneous' *in vitro* antibody production was inversely proportional to the level of antibody in the medium indicating that the inducer was immunologically specific and that it was subject to antibody feedback (Tew *et al.*, 1973; Tew, Greene & Makoski, 1976). Fragments or cell cultures from non-draining popliteal lymph nodes from the immunized animals did not produce antibody spontaneously *in vitro* but produced it efficiently when exogenous antigen was added (Tew & Mandel, 1978; Greene, Tew & Stavitsky, 1975). It is therefore likely that retained radioactivity is a reliable marker for persisting antigen and that such antigen plays a major role in the maintenance of immune responses.

The purpose of this study was to determine how long antigen could persist in immunized animals which possess efficient immune clearance mechanisms. The ability to mount a 'spontaneous' antibody response is, we believe, an index of antigen activity. Previous studies of such responses by one of us (Tew *et al.*, 1973) and by others (Stecher & Thorbecke, 1967; Mitchison, 1969) have indeed suggested that significant amounts of antigen can be found for periods ranging up to 18 months. Such data, however, are not in accord with the published half lives of simple protein antigens such as human serum albumin (HSA) (Ada, Nossal & Pye, 1964; Britton & Celada, 1968) which we have been studying. Most previous antigen half-life determinations have started shortly after antigen administration and the calculations include the period of rapid antigen phagocytosis by macrophages during which the bulk of the antigen is degraded. The true half-life determination for the immunologically relevant antigen localized in lymphoid follicles should begin after the bulk of the antigen has been cleared from the phagocytic cells (Tew & Mandel, 1978).

We show in this study that antigen elimination in an immune animal is biphasic and consists of an initial rapid clearance of antigen by macrophages followed by a second phase of antigen retention in lymphoid follicles. This retention results in a long half-life of the residual antigen. We also show that long-term retention of antigen can occur in some non-lymphoid tissues near the site of injection.

MATERIALS AND METHODS

Animals

CBA/H WEHI mice of both sexes, aged between 6 and 12 weeks, from the specific pathogen-free colony at The Walter and Eliza Hall Institute, were used in all experiments. The mice were primed, boosted and were generally used within the next 3 months, during which time they were held under conventional conditions. Animals injected with [^{125}I]-HSA were given water containing KI (50 mg/l) 2 days prior to injection and maintained on this water thereafter to minimize uptake of radioactivity by the thyroid gland.

Antigen

Crystallized human serum albumin (HSA) (Commonwealth Serum Laboratories, Melbourne, Australia) was used either in native form, or heat-aggregated when used for priming (Schmidke & Unanue, 1971). For certain experiments HSA was radio-iodinated using the Chloramine-T method (Greenwood, Hunter & Glover, 1963). The specific activity of the radiolabelled antigen was between 10 and 20 μCi/μg.

Immunization

Mice were primed in both hind foot pads with 0·5 mg of heat-aggregated HSA suspended in Freund's complete adjuvant (FCA) in a 50 μl volume. Two to four weeks later they were boosted in the same sites with the same amount of immunogen. The mice were used 3–12 weeks later.

Macrophage cultures

Non-immunized CBA mice were injected intraperitoneally with 1 ml of thioglycolate broth. Five days later, the mice were killed and the peritoneal cavity of each mouse was washed with 5 ml of mouse tonicity phosphate buffered saline (PBS). The macrophage-rich fluid was collected, centrifuged, and the cells were suspended in Dulbecco's modified Eagle's medium (DME) supplemented with 15% foetal calf serum

(FCS) at a concentration of 7×10^6 cells per ml. The cells were dispensed in 1 ml volumes into 10 ml sterile plastic tubes. The tubes were incubated overnight at 37° in 10% CO_2 and air in a humidified incubator. The non-adherent cells were removed and the tubes were gently washed with sterile PBS. The number of adherent cells remaining varied between 1·9 and $2{\cdot}5 \times 10^6$ cells/tube. Three micrograms of [^{125}I]-HSA (1·45 μCi/μg) was added to each tube with enough mouse anti-HSA to be at equivalence and the volume was adjusted to 1 ml with DME-FCS. The mixture was incubated at 37° for 30 min. The tubes were washed by gently filling and decanting twice with 10 ml of PBS. The medium in each tube was replaced with DME-FCS and the tubes were incubated at 37° as before. At various times, ranging from immediately after washing to 4·5 h later, pairs of tubes were centrifuged and the amount of radioactivity remaining associated with the cells was determined as well as the level of TCA precipitable and TCA non-precipitable radioactivity present in the supernatant fluid. The TCA precipitation was carried out by incubating the supernatant fluid with an equal volume of 10% TCA overnight at 4°. The resulting precipitate was collected by centrifugation and the radioactivity in the supernatant fluid and the precipitate was determined.

Autoradiography

Animals were killed by cervical dislocation. The popliteal and inguinal lymph nodes, spleen and foot pads were dissected and immediately placed in Bouin's solution. After fixation, the tissues were dehydrated and embedded in paraffin by routine techniques. Multiple sections of 5 μm thickness were cut and processed for autoradiography using Kodak NTB2 emulsion. Sections of tissues from each animal were exposed for periods ranging from 1 day to 70 days at 4° in the presence of Drierite. Sections were then developed with Dektol and stained with haematoxylin and eosin. Smears were also made on gelled slides of the peritoneal macrophages used for the *in vitro* antigen degradation studies. Duplicate smears were exposed for 9 and 20 days, developed as above, and stained with Giemsa.

Statistical methods

In all experiments, mice were used in groups of three to twelve age and sex matched animals. When both legs or popliteal nodes from a single animal were treated identically, the data for both sides were averaged and used as a single value. Standard statistical methods were used to calculate the arithmetic mean and standard error of the mean. The antigen half-life was estimated from the slope of the least squares regression of the logarithm of antigen retained against time. The 95% confidence interval was similarly derived from the 95% confidence interval of the slope.

RESULTS

Antigen half-life at the site of injection and in the draining lymph node

The kinetics of antigen clearance from the feet of immunized mice injected in the hind foot pads with [^{125}I]-HSA was monitored using a gamma counter fitted with a small mobile probe (Fig. 1). The injected [^{125}I]-HSA disseminated from the injection site rapidly. By the end of the first 24 h, over 99% of the radioactivity had been cleared from the hind foot. Antigen continued to be cleared rapidly during the next 3 days although a marked change in the rate of

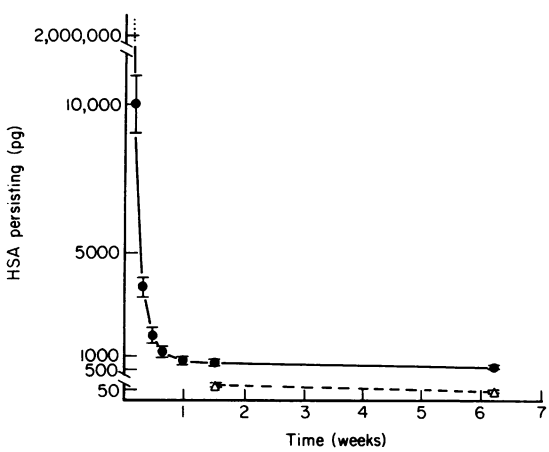

Figure 1. The rate of clearance of [^{125}I]-HSA from the hind feet (•) and popliteal lymph nodes (▵) of HSA immunized mice. A group of twelve mice was injected in both hind footpads with 2·5 μg of [^{125}I]-HSA (11 μCi/μg). Clearance of the ^{125}I from the foot was monitored using a gamma counter fitted with a small mobile probe. On day 11, six mice were killed and the radioactivity retained in the isolated popliteal lymph nodes was determined using a low background gamma counter. On day 45 the remaining mice were killed and the amount of retained radioactivity was likewise determined. The relationship between c.p.m. and picograms HSA was determined using a small sample of the original [^{125}I]-HSA. This factor was used to convert persisting c.p.m. in the tissue into picograms HSA.

clearance became apparent. This change was reflected by the rapidly increasing antigen half-life values which were 3·1 h on day 1, 15 h on day 2, 30 h on day 3 and 40 h on day 4. In the experiment shown in Fig. 1 the radioactivity levels on days 7 and 10 were not statistically different and from this time onward it was necessary to allow long periods to elapse before differences could be detected. In the experiment illustrated in Fig. 1, the value on day 45 differed significantly from that recorded on day 10. The antigen half-life during this period was calculated to be 6·1 weeks with a 95% confidence interval between 3·7 and 16·6 weeks.

In the initial experiments we expected a large proportion of the radioactivity retained in the legs to be in the draining popliteal lymph nodes. These nodes, however, contained only a few per cent of the total radioactivity retained in the legs. In the experiment shown in Fig. 1 the lymph nodes removed on days 10 and 45 contained only 3–4% of the total radioactivity retained in the legs at these times. The antigen half-life in the lymph nodes determined during this period was, however, similar to the half-life in the foot. The calculated value was 8·1 weeks with a 95% confidence interval between 5·1 and 20 weeks.

Since the antigen in the nodes represents only a small proportion of the antigen retained in the leg, we also studied the kinetics of clearance from the popliteal lymph node during the first 11 days after injecting [^{125}I]-HSA (Fig. 2). The rate of clearance was essentially the same as in the foot. Rapid clearance occurred for the first few days and a marked shift towards a more stable state occurred between days 3 and 6. Autoradiography of these nodes indicated that 4 h after injection, antigen was present in the subcapsular and medullary sinuses, and in the superficial cortex as well as in the lymph node follicles (Fig. 3a and b). By 24 h, and after 3 days, most of the antigen had been cleared from the macrophages and could only be detected in these cells at long exposure times. Grains over the lymph nodes follicles, however, were prominent even with short exposure times (Fig. 3c and d). By day 6, i.e. when the shift in the retention profile was apparent, the antigen in the medulla, subcapsular sinus, and the superficial cortex had been largely eliminated and the retained antigen was confined mainly to the lymphoid follicles (Fig. 3e and f). Grains over macrophages could only be detected with very long exposure times (> 50 days) when the macrophages were lightly labelled (Fig. 3f). It should be noted that in the same section the grains over the lymph node follicle were confluent after only a few days exposure.

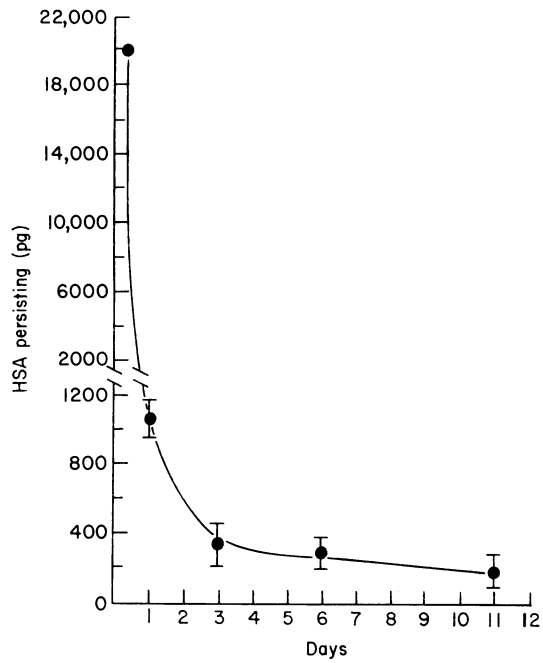

Figure 2. The rate of clearance of [^{125}I]-HSA from the popliteal lymph nodes of immunized mice during an early period after challenge. A group of fifteen mice was injected in both hind foot pads with 2·5 μg [^{125}I]-HSA (23 μCi/μg). At 4 h, 1 day, 3 days, 6 days and 11 days mice were killed in groups of three and the radioactivity retained in the popliteal lymph nodes was determined. The relationship between c.p.m. and picograms HSA was determined as in Fig. 1.

Antigen half-life in peritoneal macrophages

Examination of autoradiographs of tissue sections indicated that the clearance of antigen from macrophages was rapid. To examine quantitatively the rate of antigen catabolism by macrophages, peritoneal macrophages were allowed to ingest radiolabelled antigen in the form of antigen–antibody complexes and the rate of antigen clearance was monitored (Fig. 4). Under these *in vitro* conditions, the half-life of antigen in the macrophages was only 2 h with a 95% confidence interval between 1·5 and 3 hours. Autoradiography showed large quantities of intracellular antigen at early periods and a rapid clearance which correlated with the release of TCA non-precipitable material into the supernatant fluid (Fig. 5a and b). If macrophages *in vivo* catabolized antigen at a similar rate, it is not surprising that the radiolabel observed in lymph node macrophages at 4 h is virtually eliminated by 24 h and that radiolabel was not readily detectable in macrophages after a few days. A surprising feature

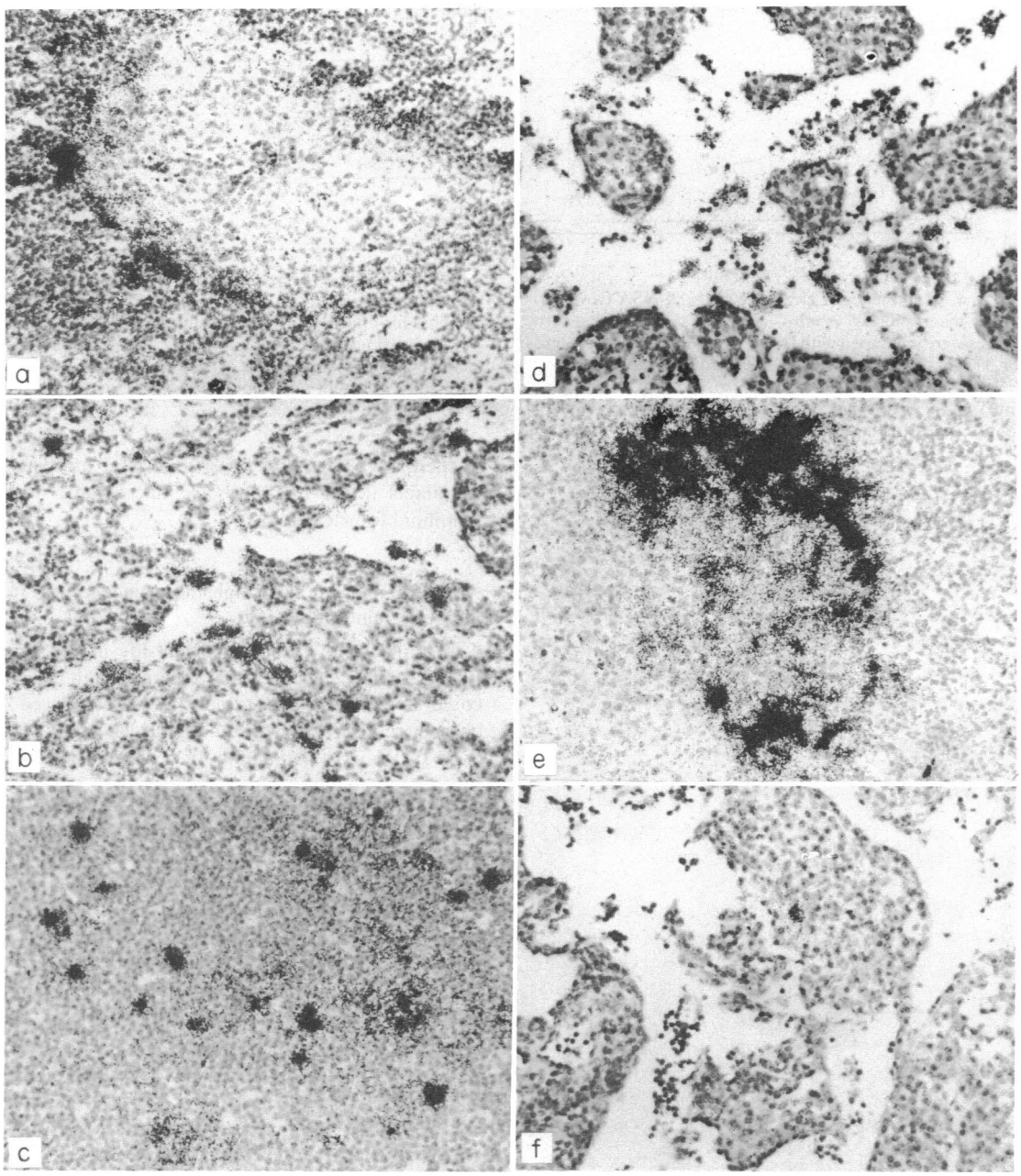

Figure 3. (a) and (b). A pair of photomicrographs from the same popliteal lymph node of an immunized mouse injected with [^{125}I]-HSA 4 h previously. (a) shows that already there is a marked localization of radioactivity in the lymphoid follicle particularly concentrated near the germinal centre. The exposure was for 8 days. By contrast, (b) shows the very heavy localization in sinus lining macrophages of the lymph node medullary cords and shows a heavy labelling density after only 1 day exposure before development. (c) and (d) are from a single popliteal lymph node of an animal given [^{125}I]-HSA 3 days previously. In this instance, (c) shows heavy focal localization in the lymphoid follicle after an exposure of only 2 days, whereas in (d), the medullary sinus lining cells are lightly labelled and required a 21 day exposure before development before a reasonably heavy grain density was seen. (e) and (f) are from an individual popliteal lymph node from a mouse given [^{125}I]-HSA 6 days previously. Both figures are from a section exposed for 54 days and show dense confluent label over cells in the follicle but a virtual absence of grains over the sinus lining cells of the lymph node medulla. All figures are of haematoxylin-eosin stained sections magnified $\times 200$.

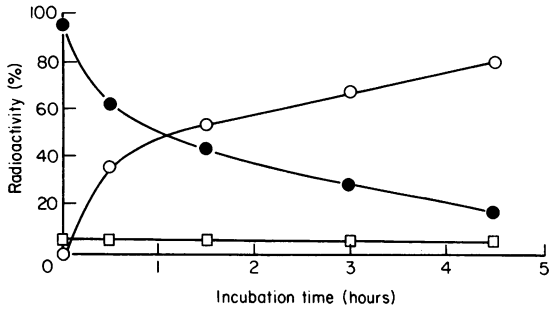

Figure 4. The kinetics of clearance of [^{125}I]-HSA from peritoneal macrophages *in vitro*. Adherent peritoneal macrophages were incubated with [^{125}I]-HSA-anti-HSA complexes for 30 min. The cells were then washed and the clearance of ^{125}I from the cells was monitored (●), as well as the appearance of TCA non-precipitable (○) and TCA precipitable counts appearing in the supernatant (□).

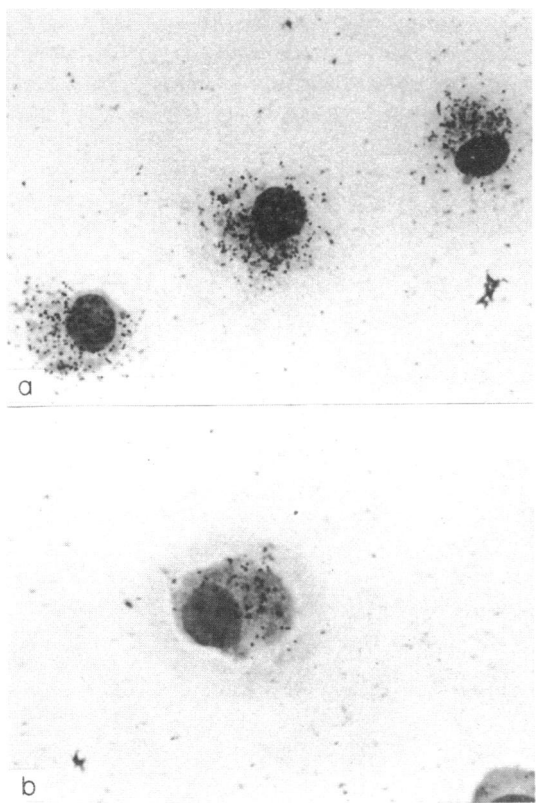

Figure 5. Photomicrographs of peritoneal macrophages given [^{125}I]-HSA-anti-HSA complexes as described in the text. (a) shows macrophages taken 30 min, and (b) cells taken 4·5 h after being given the immune complexes. Both figures are of cells exposed for 9 days before development and show that there is a marked decrease in grain counts over the cells after 4·5 h (Giemsa stained smears; magnification × 800).

Table 1. The lack of effect of foot pad amputation on the retention of antigen in the popliteal lymph nodes*

Time after amputation (days)	Ratio of Node from foot pad amputated leg† / Node from normal leg (Mean ± SE)‡
8	1·08 ± 0·24
35	0·90 ± 0·21

* Two months after boosting, the animals were injected in both hind foot pads with 2·5 μg [^{125}I]-HSA. Twelve days later one hind foot pad was removed from each animal.

† The ratio was calculated on the basis of radioactivity retained in each lymph node.

‡ Each time-point represents the mean of eight animals.

of these studies was the absence of large amounts of radiolabel in the tingible body macrophages in the lymphoid follicles.

Antigen retained in the lymph node is independent from antigen retained in the foot

The similarity of the antigen half-life in the lymph nodes and feet suggested that the antigen in the node could be turning over rapidly and be replaced by antigen released from the much larger depot in the foot. If this were happening, then amputation of the foot should, in time, result in clearance of antigen from the popliteal node. This was tested by injecting immunized mice in both hind footpads with [^{125}I]-HSA. Seventeen days later one foot was amputated through the ankle joint from each mouse. This removed between 60–80% of the total radioactivity in the leg and probably included the majority of the antigen which could anatomically be in a position to drain into the popliteal node. Five weeks after the amputation, the popliteal lymph nodes were removed, and the level of radioactivity retained in the popliteal nodes from the normal and amputated legs was compared. The ratio of radioactivity in the two nodes was unity, and did not change with time after amputation (Table 1). It therefore appears unlikely that antigen in the node is rapidly being replaced from a distal depot.

DISCUSSION

The major conclusion derived from the present study is that a simple protein antigen can be retained in an

actively immunized animal for much longer than has generally been appreciated. Previous work on retention of HSA indicated a half-life of 16 h in the popliteal lymph nodes of immunized rats (Ada *et al.*, 1964) and 16 h in the blood of normal mice (Britton & Celada, 1968). These times were calculated shortly after the antigen was injected, during which period antigen is catabolized vary rapidly. After the initial few days of rapid antigen clearance, however, a second much longer half-life becomes apparent, as is indicated in this study, by a dramatic shift in the slope of the regression line monitoring antigen clearance. At the cellular level the initial rapid clearance is associated with the catabolism of antigen by macrophages and the second slower phase of clearance appears to be associated with antigen on special follicular cells that are characterized by long dendritic processes (Nossal, Abbot, Mitchell & Lummus, 1968). Antigen associated with these dendritic cells has a half-life of approximately 2 months compared with 2 h in peritoneal macrophages. Examination of autoradiographs in the present study confirmed this differential since follicular labelling was heavy after quite brief exposure times, whereas long exposure times were required to reveal any radioactivity persisting in macrophages in nodes taken after the third day following antigen injection.

Each picogram of HSA retained in an animal represents approximately 10^7 molecules. In these studies the ratio of antigen to cells after the first week was about 30 picograms per 10^6 cells. The frequency of dendritic cells in the cell pool is not known. Judging from autoradiography and preliminary cell separation data (unpublished observations), it would appear that a reasonable estimate would be less than one radioactive cell per thousand unlabelled cells in a total lymph node population. If antigen were uniformly distributed among all dendritic cells then each radioactive cell would have about 300,000 molecules of HSA on its surface. Using the figure of $T\frac{1}{2}=2$ months each cell would still have about 5000 molecules of antigen at the end of a year. If these molecules were appropriately arranged on the surface of the dendritic cell, this amount of antigen may be able to bind enough receptors to stimulate a B cell. Certainly with the number of assumptions made in this analysis no definitive statements can be made, but it does not seem unreasonable to believe that retained antigen could play a role in immune responses for many months or even years.

In previous work (Tew & Mandel, 1978) we found that the presence of follicular antigen correlated with induction of antibody synthesis during the maintenance phase of the immune response. We therefore believe that persisting antigen may play a major role, in conjunction with an antibody feedback system, in the maintenance of serum antibody levels for long periods of time. If the 'spontaneous' response is an indication of retained antigen, then active antigen has been observed in rabbits after 18 months (Stecher & Thorbecke, 1967), and in mice after 7 months (Mitchison, 1969). Recent studies by Klaus & Humphrey (1977) and by Klaus (1978), indicate that follicular antigen plays a major role in the induction of B memory. In this context it seems plausible that follicular antigen could play a continuing role in maintenance of B memory. Indeed, each antigen exposure permitted by the antibody feedback system could result in the stimulation of B memory cells to produce antibody as well as in the stimulation necessary to replenish the B-memory cell pool.

A major assumption in this study is that persisting radiolabel represents retained antigen. This belief is supported by the observations that the retained radiolabel was not spread at random throughout the node but was restricted to particular cells in the lymphoid follicles. The radiolabel on these cells could then be specifically displaced by injecting non-radiolabelled antigen (Tew & Mandel, 1978). Furthermore, even after 12 weeks in the animal the radiolabel could be solubilized by treatment with guanidine hydrochloride and could be specifically precipitated by anti-HSA but not non-specifically by anti-egg albumin. In addition, the molecular weight of the solubilized radiolabel was indistinguishable from that of HSA originally injected (Tew, Mandel & Burgess, 1979). We therefore believe that retained radiolabel does indeed represent retained and undegraded antigen.

It is also of interest that antigen was retained near the site of injection. The biological significance of antigen retained for many months at this site remains to be established but such antigen could play a role in the maintenance of chronic inflammatory states. This possibility is currently under investigation.

ACKNOWLEDGMENTS

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