DISTRIBUTION OF HORSERADISH PEROXIDASE (HRP)-ANTI-HRP IMMUNE COMPLEXES IN MOUSE SPLEEN WITH SPECIAL REFERENCE TO FOLLICULAR DENDRITIC CELLS

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ABSTRACT

The distribution of immune complexes has been studied in mouse spleen stimulated to contain many germinal centers (GC's). Horseradish peroxidase (HRP)-anti-HRP complexes were used as an appropriately precise and sensitive model. We were primarily interested in the relative abilities of three cell types to interact with complexes: lymphocytes, macrophages, and follicular dendritic cells (FDC's). The latter are distinctive, nonendocytic, stellate cells located primarily at the transition of mantle and GC zones of 2° lymphoid follicles (Chen, L. L., J. C. Adams, and R. M. Steinman, 1978, J. Cell Biol. 77:148).

Binding of immune complexes to lymphocytes could not be visualized in situ. Macrophages avidly interiorized complexes into lysosomes, but did not retain them extracellularly. In contrast, FDC's could retain HRP-anti-HRP extracellularly under appropriate conditions, but did not endocytose them. Cytochemical reactivity accumulated progressively on FDC's 1-6 h after administration of complexes i.v., remained stable in amount and location for 1 day, and then was progressively lost over a 1- to 5-day period.

Several variables in the association of complexes with macrophages and FDC's were pursued. Only 1 μg of complexed HRP had to be administered to visualize binding to both cell types. Macrophages interiorized complexes formed in a wide range of HRP/anti-HRP ratios, while FDC's associated with complexes formed in HRP excess only. Quantitative studies with [125I]HRP-anti-HRP demonstrated that 20% of the splenic load of HRP associated with FDC's. Complexes formed with an F(ab')2 anti-HRP were distributed primarily in macrophages. When the levels of the third component of serum complement were depleted by prior treatment with cobra venom factor, uptake of complexes by macrophages was reduced some 50% whereas association with FDC's was abolished.

The fact that antigen excess complexes are retained extracellularly strengthens the idea that they are immunogenic. Finally, the association of complexes with FDC's seems to retard the entry of antigen into the GC proper.
KEY WORDS immune complex • follicular dendritic cells • germinal center • macrophage • complement

Complexes of antigen and antibody may function in many ways in the physiology of lymphoid organs. Several phenomena have been studied and, in each, cellular mechanisms proposed. The first studies centered on the ability of antibody to clear antigens from the blood stream, primarily because phagocytic cells recognize and ingest the complexes (3, 45). Then it was established that antibody can specifically inhibit the immune response to the subsequent administration of its antigen (42; reviewed in reference 43). Simple clearance of the antigen does not seem responsible (5). Antibody may inhibit by interacting directly with the responding B lymphocyte (11), or some other nonlymphoid, accessory cell (1). Immune complexes may also exert nonspecific, immunosuppressive effects (24, 30), possibly by a direct action on B lymphocytes (30). Finally, immune complexes can elicit specific responses, especially immunologic memory, i.e., the ability of a host to respond in an enhanced fashion to a second exposure to antigen (15, 20, 40). The following sequence of events is envisaged (reviewed in reference 41): special dendritic cells ("dendritic macrophages," "dendritic reticular cells") bind the complexes extracellularly (26, 27, 46); the latter then stimulate lymphocytes, inducing the formation of germinal centers (GC's); these regions of proliferating lymphocytes then give rise to memory cells. In spite of its potential importance, there has been little direct, detailed, or recent work on the distribution of immune complexes in lymphoid organs, particularly in relation to the proposed function of complexes in GC and memory development.

We recently reported on the anatomy of the GC region or 2° follicle¹ (7). This region contains at least three different groups of cells distinguishable by cytologic criteria: lymphocytes, both large and small (which from previous work, must be primarily B cells [17, 25, 31, 39]), macrophages and unusual follicular dendritic cells (FDC's). The latter are found primarily in the transition region between the mantle and GC, and appear to develop in relatively large numbers during the formation of the GC. FDC's are stellate in shape, and their cytoplasmic processes insinuate between and even around most of the other cells. FDC's are not typical macrophages in appearance or in ability to endocytose test particulates. We of course wondered whether these FDC's could retain immune complexes. Alternatively, both lymphocytes and macrophages are capable of binding immune complexes (4), and these cells are adjacent to FDC's in situ.

In this study, we look at the distribution of horseradish peroxidase (HRP)-anti-HRP immune complexes in mouse spleen, stimulated by sheep erythrocytes to generate many 2° follicles. We reasoned that a precise and sensitive EM marker, in this case the cytochemical reactivity of HRP, was needed to detect and dissect the contributions of different cell types in the binding of immune complexes in situ. In addition, we have been able to study some contributions of antibody, antibody/antigen ratios, and serum complement in the distribution of HRP-anti-HRP complexes.

MATERIALS AND METHODS

Mice

Conventionally reared, outbred Swiss mice were obtained from The Rockefeller University colony and were used in most experiments. Inbred DBA/2J (Jackson Laboratories, Bar Harbor, Me.), CD2F1 (Flow Laboratories, Dublin, Va.), and outbred, specific pathogen-free CD-1 (Charles River Breeding Laboratories, Wilmington, Mass.) mice were also examined. Mice of both sexes, weighing 20–30 g, and 8 wk to 8 mo in age were used.

Preparation of Tissue Specimens

Spleens were processed as previously described (7). Briefly, the spleens were fixed by perfusing glutaraldehyde retrogradely through the portal vein. 100- to 200-μm tissue chopper sections were rinsed overnight in buffer and then stained successively with diaminobenzidine tetrahydrochloride and H2O2 (DAB-H2O2) for 1 h at room temperature (14), 1% osmium tetroxide for 1 h on ice, and 0.25% uranyl acetate for 1½ h at room temperature. The slices were dehydrated and flat-embedded in Epon. This technique provided large areas of well-fixed and well-penetrated tissue in which the various regions of spleen were readily recognized.

Horseradish Peroxidase

Sigma Type II enzyme (Sigma Chemical Co., St. Louis, Mo.) was used throughout. In some cases, the

¹ The term secondary follicle is used to describe the composite of the GC of enlarged, proliferating lymphocytes, and its surrounding "mantle" or "corona" of primarily, small, nonproliferating lymphocytes.
HRP was radioiodinated (1 μCi/mg) with Na-125I using lactoperoxidase as previously described (32).

**Anti-HRP**

Antisera were raised in rabbits using 10 mg of HRP emulsified in Freund's adjuvant, followed by booster injections of 1–10 mg of soluble enzyme. In most cases, whole, heat-inactivated (56°C, 1/2 h), Millipore-filtered antisera (Millipore Corp., Bedford, Mass.) were used to form immune complexes and to immunize passively. The whole anti-HRP serum gave results similar to those with antisera that had been (a) centrifuged at 80,000 g for 6 h to remove aggregates, (b) precipitated with 40% ammonium sulfate to yield a crude Ig fraction, or (c) further purified by Sephadex G-200 chromatography to yield a homogeneous, 156,000 mol wt peak (48). An F(ab')2 fragment of anti-HRP was prepared by pepsin digestion in 0.1 M sodium acetate buffer, pH 4.5, for which the precipitin line was sharpest). 0.8-200 μg of immune complexes tested, which was complement fixation was detectable at the highest dose 20 h at 37°C followed by isolation of the symmetrical 106,000 mol wt peak on Sephadex G-200 columns. No complement fixation was detectable at the highest dose of HRP-F(ab')2 anti-HRP complexes tested, which was 100 times the dose of complexes formed with intact anti-HRP that produced detectable fixation.

**Immune Complexes**

Complexes were formed in the test tube by incubating HRP and anti-HRP overnight. The ratio of antigen and antibody relative to the equivalence point was determined by precipitation as previously described (33), or by immunodiffusion using serial twofold dilutions of antigen (where equivalence is defined as the ratio at which the precipitin line was sharpest). 0.8-200 μg of HRP mixed in varying ratios to anti-HRP were injected i.v. In most cases, complexes were given to mice without any further separation on the basis of size. In some experiments, however, we separated complexes formed at twofold antigen excess into three size classes: precipitates sedimenting at 600 g for 10 min (36% of total HRP), precipitates sedimenting at 134,000 g for 30 min (15% of total HRP), and the supernate (49% of total HRP).

Mice were also passively immunized with 0.1–0.5 ml of an anti-HRP serum that precipitated an equal volume of HRP 400 μg/ml, to equivalence. 24 h later, 10-100 μg HRP was given i.v. to form complexes in situ.

**Complement Studies**

Complement fixation in vitro was assessed using guinea pig complement (Cordis Laboratories Inc., Miami, Fla.) in a standard hemolytic assay (48). Serum complement in situ was depleted to <5% of control levels by treating mice withcobra venom factor (CVF; Cordis Laboratories Inc., Miami, Fla.), 6 U i.v. at 8-h intervals over 24 h. Serum C3 levels were measured by radial immunodiffusion (10), using a sheep anti-mouse C3 generously provided by Dr. Celso Bianco (State University of New York, Brooklyn, N. Y.).

**Experimental Plan**

GC's were first induced in spleen by the injection of 2 × 10⁶ sheep erythrocytes i.v. or i.p. 6-14 days before study. HRP-anti-HRP complexes were administered i.v. 6-24 h before sacrifice, in most cases. Enzyme was then visualized cytochemically at light and EM levels. In some experiments, [125I]HRP was used. Then the amount of radioactivity was measured in a gamma scintillation counter (Packard Instrument Co., Inc., Downers Grove, Ill.) either in whole spleen or after dissection of specific regions of spleen — red pulp, 2ª follicle, and other white pulp. The rationale of the dissection approach will be explained (see Results).

**RESULTS**

**Distribution of Soluble HRP**

We were able to visualize exogenous, soluble HRP in spleen cells only after the administration of large doses of enzyme (1 mg or more). As described previously (7), 1 h after i.v. HRP, granules of reaction product were seen primarily in macrophages, especially in the marginal zone and red pulp regions (Fig. 1). Granules were also scattered in smaller numbers throughout the white pulp, including the GC's when these were present (Fig. 1). By EM, reaction product was evident within intracellular vacuoles of typical macrophages, and also in lymphocytes of the white pulp (Fig. 2). No extracellular HRP was evident on any of the cell types, including FDC's. 24 h later, cytochemical reaction product due to exogenous enzyme had entirely disappeared.

**Cell Types Involved in the Retention of Preformed Complexes of HRP-Anti-HRP: Cytochemical Analysis**

Immune complexes were retained in a dramatically different fashion than soluble enzyme with respect to efficiency of capture, fate, and distribution. Thus, microgram amounts of complexed HRP were readily visualized, 24 h or more after injection, and in different locations. The most interesting situation will be considered first, i.e., mice primed 6–12 days with sheep erythrocyte to form many GC's and then challenged with complexes formed in slight antigen excess (two- to fourfold).

Reaction product of HRP-excess complexes was found in two locations. One was the marginal zone-red pulp area, where collections of reactive granules were noted by LM (Fig. 3). By EM, the granules proved to be lysosomes in typical macrophages. Connective tissue cells in the marginal
FIGURE 1 Light micrograph of the 2° follicle region 1 h after the administration of 3 mg of HRP i.v. Endogenous peroxidatic reactivity is noted in erythrocytes (R) in the red pulp sinuses, and in granulocytes (not shown here). Exogenous (HRP) reactivity is distributed as granules, mainly in macrophages (M), but also scattered throughout the mantle and germinal center regions (e.g. arrows). Follicular dendritic cells (F) are identified by large nuclei which lack heterochromatin. × 910.

FIGURE 2 Electron micrograph of the mantle region from the spleen shown in Fig. 1. Reaction product is found in lymphocyte lysosomes (short arrows) and occasionally in FDC processes (long arrow). No extracellular HRP is found. × 11,700.
FIGURE 3 Light micrograph of the 2nd follicle in a mouse 24 h after injection of HRP-anti-HRP immune complexes. The anti-HRP was a 156,000 mol wt, sephadex G200 cut of a rabbit antiserum; the complexes were formed in twofold antigen excess; and 27 μg of HRP was given i.v. Reaction product is seen in two locations and patterns. One is the marginal zone and red pulp where macrophages (M) have collections of granules. Connective tissue cells (CT) here and elsewhere are negative. HRP is also seen at the mantle-GC interface, primarily as thin streaks (arrows) which prove to be extracellular on FDCs (F). Some mantle macrophages (M) have granules of reactivity. Reactivity is sparse or absent deeper in the GC, either on FDC's (F) or in tingible body macrophages (TBM). Probably, complexes do not penetrate into this region. × 1130.
Figure 4 Low power EM of a mantle-GC transition region. This specimen was stained en bloc with DAB-H$_2$O$_2$ and uranyl acetate, but the sections were not stained with heavy metals. Cytochemical reaction product is distributed exclusively on cell processes of FDC's (arrows), "lighting up" the intricate and convoluted network which these cells form in the mantle zone. Some macrophages (Mac 1 to Mac 2) have endocytosed complexes, but others (Mac 3) show little reactivity. Reaction product is not found on the macrophage surface, or associated with lymphocytes, small (SL) or large (LL). $\times$ 3,800.

zone and red pulp, which endocytose markers such as soluble HRP, colloidal carbon, and thorium dioxide (7), did not take up immune complexes (Fig. 3). The other region with cytochemical reactivity was the mantle-GC interface, where streaks of reaction product were seen (Fig. 3). By EM, most of this reaction product was clearly extracellular on fine cell processes (Figs. 4-6). These processes arose almost entirely from FDC’s (7), and in this study we noted that the FDC was the only cell body with extracellular enzyme (Fig. 5). The thickness of extracellular reactivity varied depending on the dose of complexes injected and on the time the mouse was sacrificed, but, at all
Figures 5 and 6. Higher powers of the distribution of reaction product in mice given complexes in antigen excess. The material was stained en bloc only. In Fig. 5 (× 6,200), the FDC proves to be the only cell body showing extracellular stain. However, little endocytosis of HRP, e.g., in the Golgi region (arrow), is evident. In Fig. 6 (× 9,000), reactive FDC processes (arrowhead) are found near a tingible body macrophage in the mantle. This macrophage has intracellular enzyme. Even the tingible bodies (T) are reactive, but the cell surface (long arrows) is negative. Most tingible bodies, especially those deep in the GC, are negative, however.
times, labeled intracellular vacuoles in FDC's were rare. Some macrophages in the mantle contained reactive granules (Figs. 4 and 6), while others were negative (Fig. 4), even though they were adjacent to reactive FDC processes. Macrophages lacked surface staining. Binding and/or uptake of enzyme by lymphocytes was not clearly evident, although reactive FDC processes were usually adjacent to the lymphocyte surface. Extracellular enzyme on FDC processes was seen only in the GC region of spleen, and was evident in all GC's.

Complexes in antibody excess (two- to fourfold by weight) were also retained efficiently, i.e., microgram doses of enzyme produced abundant cytochemical reaction product. However, staining was entirely confined to macrophages in the red pulp, marginal zone, and to a lesser extent, white pulp (Figs. 7 and 8). Extracellular enzyme on FDC's was totally lacking even though the anatomy of the follicle region was similar to that in mice retaining antigen excess complexes. Complexes formed at equivalence were found only in small amounts extracellularly and were abundant in macrophages. Complexes formed in 10-fold antigen excess were not retained in a sufficient extent to be visualized cytochemically.

**Distribution of Preformed \[^{125}I\]HRP-Anti-HRP Complexes:**

**Quantitative Studies**

A dissection approach was devised to measure the amount of HRP present in regions of spleen rich in macrophages (red pulp-marginal zone) vs. FDC's (mantle-GC interface in 2nd follicles). \[^{125}I\]HRP was included in the immune complexes, and then cytochemistry was applied to tissue chopper slices to delineate various regions of the spleen. When slices were stained with DAB-H₂O₂, endogenous peroxidatic activity (erythrocyte) clearly distinguished red from white pulp (Fig. 9). In addition, slices from animals given complexes in two- to fourfold antigen excess had readily detectable "smiles" of reaction product in the white pulp nodules (Fig. 9a). Smiles were lacking in animals given complexes in antibody excess, or soluble enzyme (Fig. 9b). By microscope analysis, the smiles represented HRP in the mantle-GC region, which was predominantly extracellular on FDC's (Figs. 3-6). So we could then dissect out pieces of white pulp containing smiles and compare them to the remainder of white and red pulp.

18-24 h after administration of complexes in antigen excess, 0.05-0.20% of the injected HRP was recovered in spleen and 5-8% in liver. \[^{125}I\] counts/mg tissue (specific activity) was highest in the smile region (Table I). Because the smile accounted for only a part of the dissected area, the specific activity actually was much higher. Red pulp-marginal zone pieces also had high levels of radioactivity (similar to that seen in liver pieces), but the remaining white pulp had few counts.

To obtain a measure of total \[^{125}I\]HRP in various regions of spleen, we first considered white pulp counts to be background, because HRP was not visualized in white pulp aside from the mantle-GC interface. All areas of white pulp with smiles were dissected out from a large proportion of spleen slices. This amount of radioactivity was subtracted from that in whole spleen slices to give the percentage of radioactivity in red pulp-marginal zone. Some 20 and 80%, respectively, of the splenic load of HRP was then found to be associated with FDC's (smiles) and macrophages (red pulp-marginal zone), respectively (Table I).

**Kinetics of Accumulation and Loss of Immune Complexes**

The distribution of two times antigen excess complexes was examined by cytochemistry at varying times after administration of a single dose (48 μg HRP) i.v. At 1 h, enzyme was found in macrophages. The amount of cytochemical reaction product was similar to that observed over the subsequent 24 h. Extracellular HRP was rare at 1 h. At 2 h, extracellular enzyme became detectable, but selectively, on FDC's. FDC staining then increased steadily at 4 and 6 h, changed little over the remainder of the 1st day, and then was progressively lost over the next 2-5 days. Macrophage staining was also lost over this time period. If anything, reactivity was noted at later intervals in some mantle macrophages. Conceivably, the latter slowly cleared the FDC surface by phagocytosis.

Although clearly extracellular, complexes associated with FDC's appeared unable to move freely throughout the 2nd follicle. At early time points, many macrophages in the mantle near FDC's lacked detectable enzyme. At later times, little intra- or extracellular HRP was noted in the bulk of the GC even though FDC's and macrophages...
Figures 7 and 8. Light (× 890) and electron (× 4,600) micrographs 24 h after injection of 20 μg of HRP in fourfold antibody excess. Red pulp and marginal zone macrophages (M) are full of reactive lysosomes, but, again, macrophage extracellular staining is absent. Extracellular reactivity is not found on FDC's. (F).
Distribution of HRP in Passively Immunized Mice

Preloading the mouse with rabbit anti-HRP, in this case by passive immunization, led to a dramatic increase in the ability of spleen to retain a subsequent injection of soluble HRP, as has been documented in other studies (21, 36). These mice contained enzyme both in macrophages and on FDC's, as occurs in mice given preformed complexes in antigen excess.

Other Factors in the Retention of Immune Complexes

Extracellular HRP was detected over the full dose range studied (0.8-200 μg of HRP complexed in antigen excess). The number of stained FDC processes, as well as the amount of stain per process, increased with increasing doses.

Large precipitates of Ag excess complexes (sedimenting at 600 g for 10 min) localized predominantly in macrophages in spleen. Smaller aggregates (sedimenting at 134,000 g for 300 min) were retained extracellularly on FDC's primarily.

The soluble component in the preparation of antigen excess complexes (i.e., not sedimenting at 134,000 g for 300 min) was not detectable at the doses used, 165 μg of HRP.

Complexes formed with an F(ab')2 anti-HRP in two times HRP excess were also retained. The dose of complexes had to be increased 5- to 10-fold over complexes formed with intact anti-HRP to achieve comparable levels of cytochemical reactivity. Most of the reaction product in F(ab')2 complexes was present in macrophages, including those in the mantle region (Fig. 10). By EM, surface staining of FDC's was still detectable, however (Fig. 11).
TABLE I

Distribution of [125I]HRP-Anti-HRP Complexes in Spleen

| Region of spleen          | Exp 1 | Exp 2 | Exp 3 |
|---------------------------|-------|-------|-------|
| sp act (~1 cpm/mg dry weight) |       |       |       |
| Whole spleen              | 401   | 147   | 159   |
| Mantle-GC interface*     | 621   | 308   | 249   |
| White pulp‡              | 77    | 30    | 24    |
| Red pulp                  | 455   | 179   | 183   |

Total activity (% of total cpm in spleen)

| Zone                        | Exp 1 | Exp 2 | Exp 3 |
|-----------------------------|-------|-------|-------|
| Red pulp-marginal zone      | 78    | 83    | 79    |
| Mantle-GC interface         | 22    | 17    | 21    |

[125I]HRP complexes in twofold HRP excess were injected into individual mice 24 h (Exp 1) and 18 h (Exp 2 and 3) before sacrifice. Spleens were perfusion-fixed, and tissue chopper slices were stained with DAB-H2O2. Specific regions of the spleen were first dissected out (see Results and Fig. 9) with 21-26 gauge hypodermic needles, washed extensively to remove soluble radioactivity, and then groups of 50-100 pieces were assayed for radioactivity/dry weight. To determine total activity in red pulp-marginal zone vs. mantle-GC interface, we first considered the value for white pulp as background. We then obtained a value for total activity in a large sample of spleens and subtracted this from whole spleen data to obtain a value for red pulp-marginal zone.

* Mantle-GC interface: This is the region identified as a smile on tissue chopper sections (Fig. 9a) and containing predominantly extracellular HRP or FDC's.
‡ White pulp: This is the middle of the nodule without any smile and without any cytochemical reaction product by microscopy.

DISCUSSION

HRP as a Model Antigen

The glycoprotein enzyme, HRP, has several advantages as a model for studying the distribution of antigenic materials in lymphoid organs. Both antigen (HRP) and antibody are readily available. The enzyme can be visualized cytochemically at LM and EM levels. The technique (14) can be applied to aldehyde-fixed tissue, so that the complex microanatomy of lymphoid organs is maintained. Precision is gained from the fact that the highly insoluble reaction product appears to be deposited very close to the enzyme’s active site. Sensitivity is indicated by the fact that we readily visualized HRP in spleen when we injected <1 μg of complexed enzyme i.v., under conditions in which only 0.2% of the injected antigen was present in the entire spleen.

The need for a precise and sensitive marker such as HRP became evident after several developments that occurred after the initial work on antigen retention in lymphoid organs. First, it is now known that B lymphocytes bind immune complexes (4, 12), and it has also been suggested that typical phagocytic cells can both ingest and retain antigens extracellularly (44). Both B lymphocytes and macrophages are present in lymphoid follicles, so that their potential contribution to antigen retention must be considered. In addition, the complex microanatomy of the 2° follicle was not outlined in earlier work, especially the unique cytologic features of FDC's. These cells send out fine cell processes that make contact with most other cells (lymphocytes and macrophages) in the follicle, especially at the mantle-GC interface (7).

Studies on the Role of Serum Complement

Mice containing GC's were treated with CVF to decrease serum C3 levels to <5% of control values. This treatment did not alter the anatomy of the follicular region, particularly the FDC's. However, antigen excess complexes formed with intact or with F(ab')2 anti-HRP were now visualized only in macrophages, and not extracellularly on FDC's.

To gain some idea of the clearance and total amount of HRP reaching the spleen, in CVF mice, the organ distribution of [125I]HRP anti-HRP complexes in twofold HRP excess was followed in two control and three CVF mice (these controls behaved like several others in independent experiments). Complexes were cleared rapidly from the circulation in both groups (Table II) although a little more rapidly in CVF mice. Liver uptake was increased slightly after CVF treatment, and splenic uptake was reduced to 30-40% of control (Table II).

Because complement appeared to be required for FDC retention, we compared the ability of complexes formed at varying HRP/anti-HRP ratios to fix guinea pig complement in the test tube by a hemolytic assay. All complexes fixed complement similarly in vitro even though complexes in antigen excess preferentially associated with FDC's in situ. We conclude that complement fixation alone may be insufficient to mediate binding of FDC's. Some additional step must occur (see Discussion).
Figures 10 and 11 Light (× 1,210) and electron (× 4,100) micrographs after injection of 40 μg of HRP as a twofold antigen-excess complex formed with an F(ab')2 anti-HRP. In Fig. 10, extensive uptake by macrophages (M) in both mantle and marginal zone is evident. Extracellular staining is barely detectable by LM, though by EM (Fig. 11) it is evident (arrows) exclusively on FDC's. Granulocytes (G) are infrequent in the mantle zone.
Organ Distribution of $^{[125]}$HRP-anti-HRP after CVF Treatment

| Organ     | Control Mouse 1 | Control Mouse 2 | CVF-Treated Mouse 1 | CVF-Treated Mouse 2 | CVF-Treated Mouse 3 |
|-----------|----------------|----------------|---------------------|--------------------|--------------------|
| Blood (50 µl) |                |                |                     |                    |                    |
| 1 h       | 21.3           | 25.8           | 15.7                | 18.9               | 8.47               |
| 3 h       | 8.61           | 13.7           | 4.34                | 4.89               | 3.10               |
| 5 h       | 3.24           | 4.24           | 2.54                | 2.72               | 2.34               |
| Liver     | 363            | 368            | 477                 | 467                | 489                |
| Spleen    | 15.6           | 16.1           | 7.57                | 7.73               | 6.29               |
| Lung      | 11.0           | 6.66           | 5.75                | 4.18               | 4.39               |

Total cpm/organ ($\times 10^4$)

$6 \times 10^6$ cpm of $^{[125]}$HRP in a twofold antigen excess complex was injected i.v. into two control and three mice treated previously with CVF, to reduce serum C3 levels to <5% of control. At 1, 3, and 5 h, 50-µl blood samples were taken, and at 6 h the mice were sacrificed. Total cpm is presented for liver, spleen, and lung. The average weight of liver, spleen, and lung was 1.6, 0.14, and 0.22 g. 55-65% of the counts were precipitable in 10% TCA.

Extracellular, what cell types are involved in antigen binding, and whether the marker is truly associated with the surface of a particular cell or simply distributed at random in the extracellular space.

The attributes of HRP cytochemistry were not available at the time of previous studies on the distribution of antigens. Immunofluorescence has been used to localize antigens (45, 46), but the material consisted of relatively thick sections. Autoradiography, primarily at the light level, has also been used, especially with radioiodinated antigens (27). This approach is simply not precise enough for establishing the cellular distribution of small amounts of antigen. EM autoradiography of $^{[125]}$I-tagged antigens was attempted to gain increased resolution (26, 38), but then large doses of radioiodinated antigens and/or heavily substituted materials were required, further complicating the interpretation of the observations.

Another potential advantage of HRP, not really utilized in this study, is that this enzyme is capable of actively inducing immune responses in which the specific antibody-forming cells can be visualized (2). In this paper, we have looked at the distribution of immune complexes formed in vitro or after passive immunization in situ. It remains to be seen whether our observations can be extended to mice undergoing an active immune response to HRP.

Cell Types Involved in HRP Retention

Most cell types in lymphoid organs could interact with HRP, depending on the form and amount of enzyme administered. After large doses (milligrams) of soluble enzyme, lymphocytes both large and small showed reactive intracellular granules which had to be derived by endocytosis of HRP in the extracellular space. HRP has a small molecular weight (40,000) and is rapidly cleared from circulation, especially by glomerular filtration (37). Possibly, endocytosis of lower doses of HRP would have been detectable had the life span of circulating enzyme been longer. It is unclear to what extent other soluble protein antigens might be pinocytosed the same as HRP. If HRP is interiorized in the content of the fluid phase, then all other proteins might be expected to be included in lymphocyte endocytic vacuoles. Alternatively, HRP might have been interiorized by a more selective, adsorptive pathway.

Macrophages, i.e., mononuclear cells with a well-developed vacuolar system including abundant lysosomes, actively interiorized HRP administered either as soluble or complexed enzyme. Much lower doses of immune complexed HRP had to be given to detect uptake (several hundredfold), and complexed enzyme persisted intracellularly for longer periods. Persistence or retention...
of soluble and complexed HRP on the macrophage surface was not detectable. All these findings are identical to those previously documented in tissue culture of mouse peritoneal macrophages (32, 33). We therefore presume that the mechanism of uptake in situ is similar to that occurring in vitro, i.e., soluble enzyme is interiorized in the fluid phase of pinocytic droplets, and immune complexes are found and interiorized via recognition sites for the Fè region, and/or affixed C3.

The cell type of most interest was the FDC. These cells were previously shown to be nonendocytic cells, by the use of nonimmunogenic particulate tracers (7). Both colloidal carbon and thorium dioxide particles associated selectively with the FDC surface (7), and in this study immune complexes behaved similarly. A critical range of antigen to antibody had to be used, that is, two- to fourfold antigen excess, before complexes associated with FDC's; but under no circumstances was active endocytosis evident. With antigen excess complexes, the concentration of HRP in the FDC-rich areas of spleen was probably greater than in any other. We do not know how long active antigen (vs. enzyme) can remain in association with FDC's. At the doses used, extracellular enzyme activity was progressively lost over a 3- to 6-day period.

Our observations demonstrate the clear-cut differences between FDC's and macrophages. FDC's, which do not look like endocytic cells, retain complexes on their surface and do not endocytose them; the reverse is true for macrophages. No transitional or intermediate forms were evident. Although the nomenclature and literature are confusing on this point, cells that are variously referred to as "reticular" cells or macrophages probably represent many different cyologic and functional entities.

Mechanism of Immune Complex
Retention on FDC's

Antigen excess complexes associated similarly with FDC's in all GC's, whether elicited by the non-cross-reacting antigen, sheep erythrocyte, or by endogenous stimuli in the environment of certain batches of mice. Thus, the activity of FDC’s seems to be selective for complexes rather than the antigen per se, and corresponds to the binding of nonimmunogenic particulates documented previously (7). We are currently examining the cyologic changes that occur during the development of GC's, with emphasis on FDC's and immune complex retention. Preliminary findings are that selective binding to similar cells can occur before GC formation.

Two sorts of experiments were attempted to find out what components of an immune complex were responsible for binding to FDC's. Earlier workers emphasized the requirement for specific antibody, probably the Fè portion (16, 19, 21). We found that F(ab')2-anti-HRP-HRP complexes exhibited greatly reduced association with FDC's. We hesitate at this time to conclude that the Fè portion of the anti-HRP is critical. Naturally occurring anti-F(ab')2 antibodies (28, 49) have been demonstrated in some species. The presence of such reagents in our mice might alter the ratio of antibody to antigen in the complex and make it behave more like a complex in antibody excess.

Recent work has focused on the function of serum complement components, especially C3, in the phenomenon of antigen retention (18, 29). It has been noted that C3 is present in the same sites as antibody in cryostat sections stained with an anti-C3 reagent (13), and that depletion of serum C3 with CVF reduces either GC formation and/or antigen retention (29, 47). We found that CVF treatment dramatically reduced FDC binding of HRP-anti-HRP complexes.

Complement may operate in several ways, though it is not clear that any of those mechanisms accounts for the requirement of complexes in antigen excess. For example, complement may serve to solubilize and/or break down the complexes to smaller sizes (4, 9). This might enhance penetration of complexes into the white pulp and/or retard binding and uptake by macrophages. Complement may mediate the interaction of complexes with other cells, e.g., B lymphocytes (6), platelets, and neutrophils (4, 8), which then may help deliver and/or alter the complex so that it binds to FDC's. Finally, complement may be recognized by receptors on the FDC surface. However, none of the Fè and C3 receptors that function to bind complexes to cell surfaces in vitro (e.g., mononuclear phagocytes, lymphocytes, platelets) have been shown to mediate the prolonged retention of immune complexes on cell surfaces (e.g. reference 23). Also, we think that FDC's may arise from a population of dendritic cells that we have identified in vitro in mouse spleen suspensions (34), and these cells do not demonstrate receptors for immune complexes (35) even after prolonged culture (R. Steinman, unpublished observations).
Significance of Immune Complexes on FDC's

Ever since it was noted that antigens were retained for prolonged periods in the 2nd follicle, it has been assumed that the retained material has some immunogenic functions, e.g., triggering the proliferation of lymphocytes in the GC proper. This possibility is enhanced by our observation that complexes in antigen excess may be preferentially retained, and that the area in which complexes are deposited is the site through which most B lymphocytes recirculate (17, 25). Immunogenicity remains to be established. For example, we would like to know whether one can correlate extracellular HRP retention with the development of an immune response to HRP.

Another possible role for FDC binding is that the FDC is removing complexes from lymphocytes, and/or preventing them from gaining access to the GC. We were struck by the fact that soluble HRP within 1 h was endocytosed by GC lymphoblasts. Even at 24 h, immune complexes were localized at the mantle-GC interface with little entry into the GC. Binding of immune complexes to lymphocytes may alter their physiologic function, e.g., their ability to recirculate through lymphoid organs or to respond to antigens. These possibilities can be approached in the HRP-anti-HRP system, because the effects of the immune complexes on immune responses can be correlated with precise information on their distribution in the lymphoid organ.

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