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CELLULAR REACTIONS TO SOLUBLE FOREIGN MATERIALS IN THE RABBIT KNEE JOINT**

For well over half a century, since the days of the imaginative and enthusiastic genius, Metchnikoff, it has been known that foreign materials cause inflammation.[1] When the material is a splinter in our finger, we take it for granted that the body will mobilize its phagocytes to react to the foreign material. Less well known is that soluble foreign proteins and polysaccharides ("antigens" in usual biologic reference) regularly induce a similar prompt outpouring of polymorphonuclear leukocytes. The mechanism of this local reaction, which has been termed "norergic" as contrasted with "allergic" inflammation,[2] has received surprisingly little attention when contrasted to the vast literature on the immunologic response to foreign materials. Lack of curiosity about the mechanism of this inflammatory reaction probably stems from its generally mild and transient nature. However, this is not always true since biologists who use casein or glycogen to produce a polymorphonuclear peritoneal exudate are taking advantage, perhaps, of this type of reaction to foreign material.

During the course of some observations on inflammation and infection in rabbit suprapatellar bursae, Metchnikoff's cellular reaction to foreignness, so well described by Clark and Clark 30 years ago,[3] was re-encountered. Synovial membrane seems a particularly suitable site for observations on cellular inflammatory exudate. The observations were conducted in an effort to understand more about the nature of the acute inflammatory response to soluble materials, as well as its possible relation to the immune response elicited by these materials.

MATERIALS AND METHODS

Albino rabbits of both sexes, weighing 3 to 4 kg., were used in the experiments. Materials to be studied were undiluted or dissolved in 0.5 ml. of 0.9 per cent NaCl, and were injected with a 21 gauge needle into the suprapatellar bursae of rabbits

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anesthesized with intravenous pentobarbital (approximately 60 mg.). At appropriate times thereafter the animals were either anesthesized or killed with pentobarbital, and their suprapatellar bursae re-entered with a 19 gauge needle attached to a 2 ml. plastic syringe filled with saline. The bursae were aspirated and then washed back and forth by distending with the saline from the syringe, and during the washing process the bursae were gently manipulated to express large aggregates of exudate. If histologic sections were desired, the bursae were immediately removed and fixed in formalin. The bursal washings usually yielded little more than the 2 ml. of fluid used for the irrigation. Cell counts were obtained with a Coulter electronic particle counter, after preliminary studies had revealed an excellent correlation with conventional methods of leukocyte counting. Total exudate cells were calculated from the white cell count and the volume of material obtained from the washed synovial cavity. Differential white cell counts were obtained by centrifuging the exudate, decanting the saline, and resuspending the cell buttons in a few drops of rabbit serum. The resuspended sediment was then brushed onto coverslips and stained with Wright's stain.

Scrupulous care was exercised to prevent contamination, and all materials used for injection into the bursae were pyrogen-free, since other experiments had indicated that as little as .00005 micrograms of purified bacterial endotoxin produces a brisk inflammatory response in the rabbit knee joint.[4] The proteins studied are described in the text, but all were either obtained in sterile, pyrogen-free manner from the donor, were biologic materials intended for parenteral use in humans, or were tested for pyrogenicity intravenously in rabbits by injecting at least 100 times the intrasynovial dose.

In studies of synovial responses in immunized animals, immunity was produced by incorporation of the antigen in Freund's adjuvant, or in some instances by repeated intravenous or intraperitoneal administration of the antigen.

RESULTS

A. Synovial inflammatory response to saline and rabbit serum

Simply washing the synovial cavity with saline yielded total cell counts of less than 100,000, mainly small lymphocytes or synovial lining cells. However, since the inflammatory effects of protein and polysaccharide solutions were to be examined at periods of several hours after injection into the suprapatellar bursae, an injection of saline into the opposite knee was usually employed as a control for the inflammatory response to the injection itself and of the distension of the bursae produced by the 0.5 ml. volume. Thirty-two such control injections with saline, with cell harvest six hours later, were performed during the course of experiments with protein solutions. A definite inflammatory response was detected. In the 32 saline-injected synovial cavities, the six-hour total cell count varied from 300,000 to 6,300,000 cells, almost entirely polymorphonuclear leukocytes. The mean count was 1,950,000 cells, with a deviation of 1,410,000. The 95 per cent confidence limit extended to a total of 4,700,000 cells.

Since many of the experiments involved heterologous whole serum or protein solutions with potential osmotic effects, injections of autologous or homologous rabbit sera were studied as well. The response to autologous or homologous rabbit sera was similar to that with saline; in 38 injections from six experiments, the six-hour exudate contained from 900,000 to 4,600,000 cells, predominantly polymorphonuclear leukocytes.

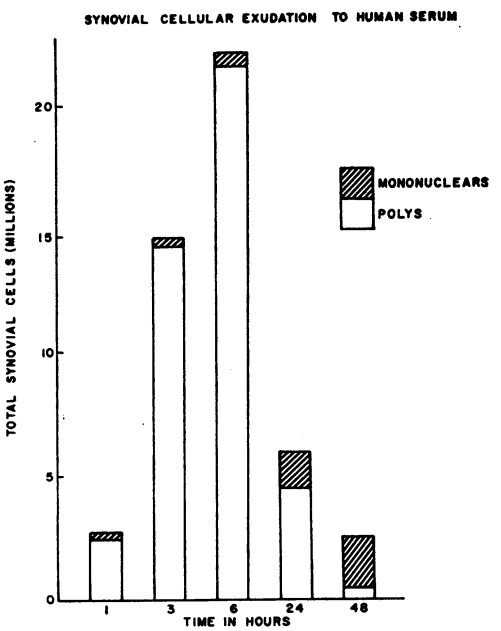

FIG. 1. Cellular responses in rabbit synovial cavities at intervals after injection of 0.5 ml. of human serum.

In a few experiments in which the bursae were simply entered with a needle and washed six hours later, the exudate was similar to the saline response. It seems probable, therefore, that the saline and rabbit serum responses represent little more than the effects of the trauma of injection.

B. Inflammatory response to heterologous serum

Heterologous serum was used to study the synovial inflammatory response, since it was felt that such material would not be a primary irritant by virtue of its pH or other physical characteristics, and since the rabbit serum observations provided appropriate control data. In Figure 1, the sequence of the inflammatory response was followed by harvesting the

synovial exudate from two rabbits (four joints) at intervals of 1 to 48 hours after intrabursal injection of 0.5 ml. of human serum. At one hour, there was little change except for an increase in the percentage of polymorphonuclear leukocytes, but a marked granulocytic reaction occurred by six hours. At 24 hours, the reaction was subsiding and more large mononuclear cells were appearing. By 48 hours, the total count and percentage of

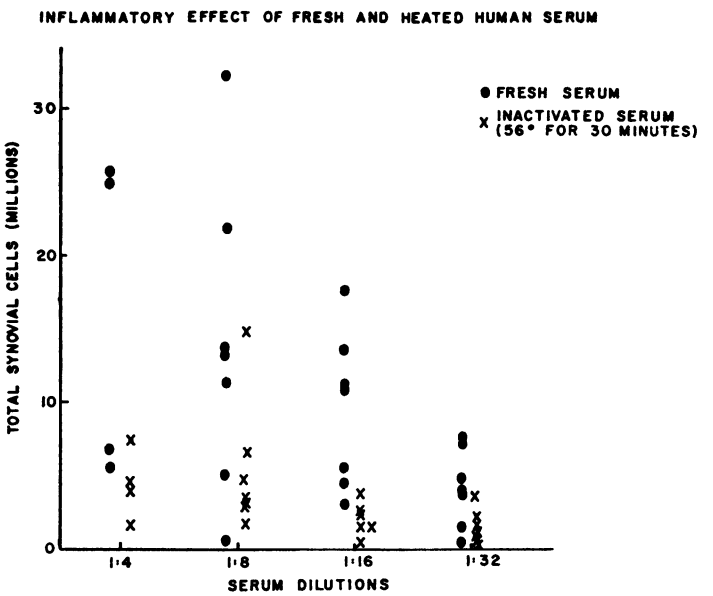

FIG. 2. Comparative synovial inflammatory effect of the same human serum fresh (•) and heat-inactivated (X).

polymorphonuclear cells were markedly diminished, and the mononuclear cells consisted of large phagocytic macrophages. Mouse serum gave similar responses.

Because complement or other heat-labile proteins have been reported to have some role in inflammation,[5] fresh human serum was compared with serum heated to 56° C. for 30 minutes. Serial dilutions 1:4; 1:8; 1:16; 1:32 were made with fresh and inactivated sera, and their inflammatory capacity measured six hours after intrasynovial injection. The results are shown in Figure 2. As indicated in the figure, the heat-inactivated human serum in various dilutions generally produced less inflammation. Statistically different results were noted in the 1:16 and 1:32 serum dilutions ($p < .001$ and .05, respectively).

*C. Inflammatory response to some purified materials**

Selected purified, pyrogen-free proteins and mucopolysaccharides were injected into the knee cavity, and their ability to produce inflammation within six hours assayed. The materials selected included traditional antigens in rabbits (human serum albumin and globulin, ovalbumin, and bovine serum albumin) and other materials that are poorly if at all antigenic (human hemoglobin, dextran, gelatin).

Ovalbumin, the material used in many of the experiments, produced inflammatory cell responses similar in time sequence to the response observed with heterologous whole serum. As seen in Figure 3 the common antigens (human albumin, human globulin, ovalbumin, and bovine albumin) all produced inflammatory responses six hours after intrasynovial injection, while dextran and gelatin did not cause polymorphonuclear exudation. Human hemoglobin, in hypertonic concentrations of 15 gm/100 ml. produced a minimal inflammatory response, very similar to that produced by the same concentrations of homologous (rabbit) hemoglobin. The data in Figure 3 are with relatively large amounts of material (five milligrams or more) and although complete titrations of the reaction were not performed with all reactive materials, an inflammatory response was usually produced by one milligram, with only a rare animal exhibiting an unmistakable inflammatory response to less than 100 micrograms of the foreign material. Human serum albumin produced less reaction than the other antigenic materials.

D. Exudative response in immunized animals

The synovial inflammatory response was examined in animals immunized with ovalbumin, human serum albumin, and whole human serum. Table 1 shows the individual synovial exudate response in animals that had been extensively immunized with ovalbumin. Immunization consisted of injections of 5 mg. of ovalbumin in 1 ml. of Freund's complete adjuvant (Difco) in each of the four legs; two weeks later the injections were repeated. Beginning two weeks after the second injection of antigen in adjuvant, intravenous injections of 5 mg. of ovalbumin in saline were given twice a week for four weeks. Before the experiment, serum antibody content was assayed by the quantitative precipitin test of Heidelberger, *et al.*[9] One milliliter of serum was successively absorbed with 100 μg.

* Human serum albumin, E. R. Squibb & Co.; Human gamma globulin, Parke-Davis & Co.; Ovalbumin, 2x re-crystallized, Worthington Biochemical Co.; Bovine serum albumin, Pentex, Inc.; Dextran "Intradex," Glaxco, Ltd.; Special gelatin solution, Knox Gelatine Products.

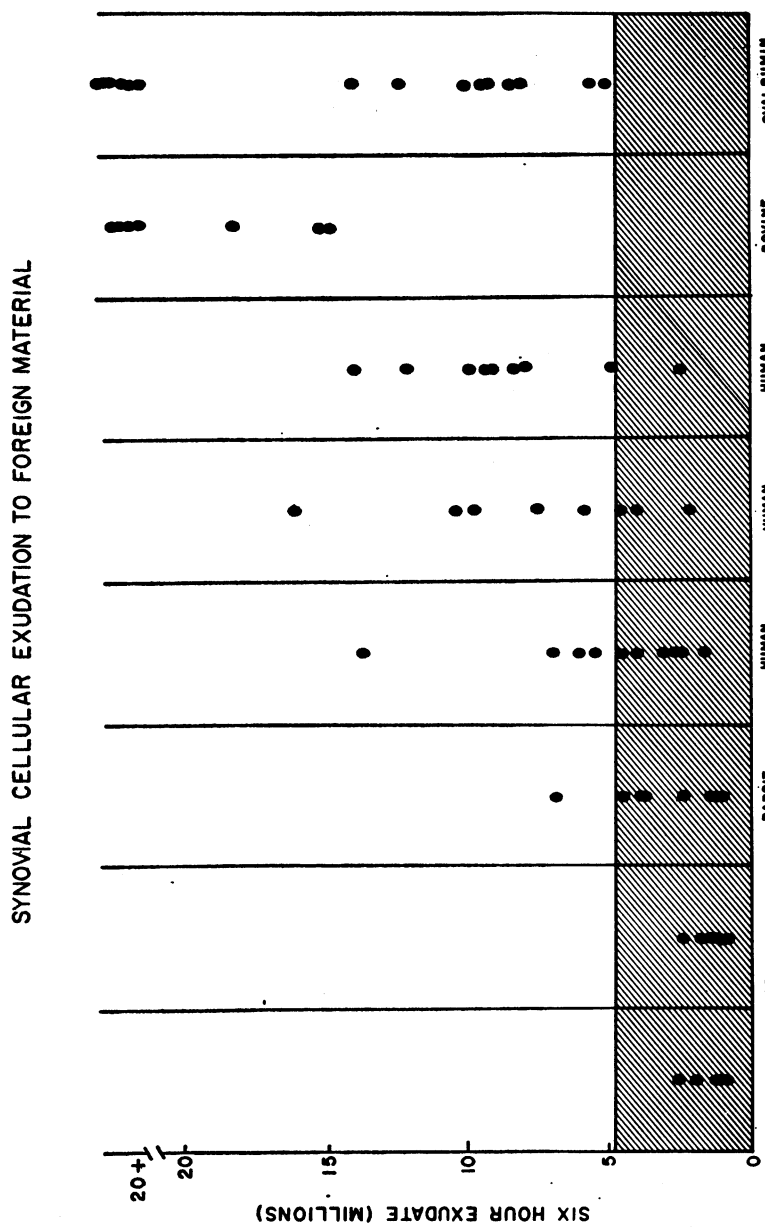

FIG. 3. Synovial inflammatory reaction produced by a variety of soluble foreign materials six hours after injection into rabbit suprapatellar bursae.

of ovalbumin in 0.1 ml. of saline. Sera from the seven immunized rabbits required 300-500 μg. of ovalbumin for complete precipitation of antibody, giving an estimated serum antibody nitrogen content of 330-560 μg./ml.

The table shows that the immunized animals reacted with extensive polymorphonuclear exudate to both 0.5 and .05 mg. of ovalbumin in 0.5 ml.

TABLE 1. COMPARISON OF THE SYNOVIAL INFLAMMATORY RESPONSE OF TWO DIFFERENT AMOUNTS (0.5 MG. AND 0.05 MG.) OF OVALBUMIN INJECTED INTO THE SUPRAPATELLAR BURSAE OF IMMUNIZED AND NORMAL RABBITS. ANIMALS WERE SACRIFICED AT 6 AND 24 HOURS.

	Six-hour responses			
	0.5 mg. Ovalbumin		*.05 mg. Ovalbumin*	
	Immune	*Nonimmune*	*Immune*	*Nonimmune*
	10.7 (81%)	2.8 (94%)	7.5 (88%)	0.6 (62%)
	43.8 (95%)	1.6 (84%)	6.1 (89%)	1.0 (39%)
	32.7 (93%)	4.1 (88%)	30.0 (90%)	1.5 (72%)
	71.6 (98%)	1.2 (75%)	17.2 (87%)	0.6 (94%)
Mean	39.7	2.4	12.7	0.9
	Twenty-four hour responses			
	0.5 mg. Ovalbumin		*.05 mg. Ovalbumin*	
	Immune	*Nonimmune*	*Immune*	*Nonimmune*
	121.0 (79%)	6.3 (43%)	45.0 (86%)	1.0 (25%)
	88.0 (89%)	3.1 (22%)	73.0 (87%)	0.6 (39%)
	119.0 (80%)	0.8 (42%)	37.0 (94%)	0.5 (6%)
Mean	109.3	3.4	51.7	0.7

Numbers represent the total synovial cell response in millions, with per cent of polynuclear cells in parentheses.

of saline. This reaction was greater at 24 hours than at 6 hours. In contrast, 0.5 mg. of ovalbumin produced only a mild outpouring of granulocytes in the normal animal and the .05 mg. amount was probably no greater stimulus than saline alone.

This enhanced cellular exudate was found in rabbits immunized to human serum albumin, and was mirrored by biologic evidence of acute inflammation in the synovium, much more pronounced than the reaction in normal rabbits. Surprisingly, however, the cellular exudate to whole human serum was markedly suppressed in immune animals. This discrepancy was not explained satisfactorily, but the morphologic reaction to

whole human serum in the synovium of immunized animals was much more severe than that encountered in the ovalbumin-immune experiment, with intense vasculitis and vascular occlusions. Perhaps the intense reactions in the deeper tissues precluded the migration of granulocytes into the synovial cavity, perhaps antibodies to some of the proteins involved in coagulation may have altered the exudative response, or perhaps the quantity of antigen, which was not varied, may be a controlling factor. No attempt was made to evaluate these or other possible mechanisms.

E. Studies with I-^{131}-labeled human serum albumin in normal and immune rabbits

Commercially available I-^{131}-labeled human serum albumin (E. R. Squibb Co.) was used to produce an inflammatory exudate in normal rabbits and to study the association between the exudative granulocytes and the protein used to produce the inflammation. The same material was used in normal and immune animals to compare the rate of antigen removal from the joint cavities..

When 0.5 cc. of one per cent I-^{131}-human serum albumin was injected into rabbit joints, a polymorphonuclear exudate averaging 12,000,000 cells/joint was produced in six hours. The radioactivity in these cells was determined after 2, 4, or 6 saline washings of aliquots of the pooled exudate. Only 0.18 per cent of the I-^{131} was cell-associated, and this amount was unchanged with repeated washings of the cells. Although this percentage seems small if considered as per cent of the total I-^{131} in the joint cavity, 0.18 per cent of the 5 mg. of albumin injected represents approximately 8×10^{13} molecules of protein associated with 12×10^{6} exudative granulocytes. Although observations in immunized animals were less systematically explored, a greater amount was apparently cell-associated, probably representing phagocytosis of antigen-antibody complexes as described by others.[7,8]

To determine whether the enhanced synovial inflammatory response in immunized animals was related to differences in the local persistence of antigen, 0.5 to 1 mg. of HSA-^{131} was injected into the knee joints of 5 normal rabbits and 5 rabbits immunized by repeated intraperitoneal injections of one per cent HSA. The radioactivity remaining in the joint cavity was determined by external monitoring with a probe-type scintillation counter. Levels of absorbed and circulating HSA-I^{131} were determined by monitoring the circulating blood over the precordium, and confirmed by counting of plasma in a well-type scintillation counter. There were no apparent differences in rate of removal of the radioactive protein from

the synovial cavity of the two groups of rabbits, but precordial radioactivity was always less in the immune animals, indicating more rapid removal of antigen from the circulation. This accelerated clearance of circulating antigen is a well-known and sensitive indicator of circulating antibody.[9] Figure 4 shows the joint and precordial counts obtained in a

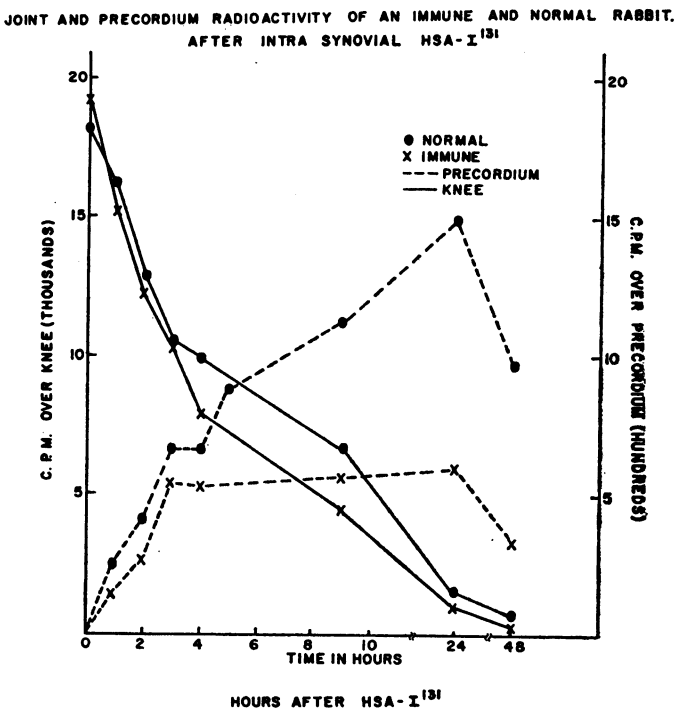

FIG. 4. Representative curves of I-[131] labeled human serum albumin removal after injection into the suprapatellar bursae of normal and immune rabbits. Although the I-[131] counted over the knees was lost at a similar rate in both animals, precordial counts were lower in the immune animal, indicative of accelerated bloodstream clearance.

representative immune and normal rabbit. Studies by others have demonstrated no difference in cutaneous antigen persistence at the local site in normal guinea pigs and those with delayed hypersensitivity.[10]

DISCUSSION

Use of the knee joint in these studies was prompted by our interest in synovial inflammation. However, the site has much to recommend it generally in studies of this type, since the technique is simple and knee joints come in pairs, while the commonly used peritoneal space cannot

provide an intrinsic control. The rabbit is convenient, since this animal has been so widely used in studies of inflammation and immunity. The synovium consists of loose vascular connective tissue with a 2 or 3 cell deep surface of large rather irregularly arranged lining cells. In non-articulating areas, villous projections increase the surface area of the synovial lining. This thin lining serves both nutritive and excretory functions for the underlying cartilage. Secretion provides the lubricant synovial fluid for the cavity, and the synovial cells remove foreign material from the cavity by phagocytosis.[11] Recent studies with the electron microscope indicate that synovial capillaries have an unusual fenestrated structure perhaps in keeping with the rapid exchange of materials by the synovium.[19] This unusual capillary structure may explain the extreme sensitivity of the rabbit knee joint to inflammation produced by endotoxin.[4] Perhaps the synovial inflammatory response to foreign proteins and polysaccharides is also unsually brisk, although comparative studies have not been done.

The materials used in this study of inflammation to foreign soluble materials were selected to avoid contamination by even small amounts of endotoxin, and the antigens were of biologic origin so that the inflammation produced was not likely to be on the basis of an acidic or basic protein that is irritative by its chemical nature. With whole serum of mouse or human origin, the intense inflammatory response is almost certainly due to some innate recognition of foreignness rather than a chemical irritant effect. This is the same interpretation that others through the years have made of this phenomenon.[1,8]

The finding of enhanced inflammation in immunized animals is in keeping with many similar observations; this is, in essence, a synovial Arthus reaction. Others have noted increased cellular exudate when antigen was injected into the peritoneum of immunized animals.[18] One feature of the cellular response was of special interest. Eosinophils have been reported in the peritoneal cavities of mice[7] and guinea pigs[14] following the intraperitoneal injection of antigen into immunized animals. In the present work, only during the late phases of the reaction to whole human serum, which produced intense vasculitis of the synovium but was not accompanied by enhanced exudation within the synovial cavity, were eosinophils detected in the exudate. Eosinophils were not seen in the other experiments, although they can be easily differentiated from the eosinophilic-staining polymorphonuclear leukocyte of the rabbit. This is in keeping with observations that the classical Arthus reaction in rabbits is a predominantly polymorphonuclear inflammatory reaction.[15] Although Cochrane, *et al.*[15] suggested that the granulocytic inflammation aided in degradation and

elimination of the antigen from the site of an Arthus reaction, our study revealed no evidence of enhanced removal of I^{-131} from the joints of immunized rabbits. Although our interpretation that removal and degradation of antigen is similar in normal and immune animals appears reasonable and fits with the quantitative observations on antigen degradation in delayed hypersensitivity,[10] it is possible that the synovial I^{-131} was attached to more degraded albumin in the immune animal or even represented I^{-131} attached to some rabbit protein.

The choice of nonpyrogenic, nonantigenic materials for study was quite limited. The dextran and gelatin solutions employed were both designed as plasma volume expanders in man, and as such are reputed to be noninflammatory and nonantigenic; the finding that they did not provoke inflammation in the rabbit synovium was to be expected. Human hemoglobin is a very poor antigen in the rabbit and precipitating antibodies can only be produced by use of adjuvants.[16] The negligible inflammatory response to this material, foreign but poorly antigenic, is of interest.

Fruhman[17] has extensively studied leukocytic mobilization to a wide range of materials in mouse peritoneum, and his observations are generally similar to these in rabbit knee joints. He points out that only endotoxin produces inflammation with minute quantities of material, and that proteins and polysaccharides produce a similar reaction with only relatively large quantities. He detected no particular pattern in the response to a number of foriegn proteins and polysaccharides.

It seems possible that there is a common mechanism by which this inflammatory reaction to foreign materials is mediated, although undoubtedly some foreign materials are also damaging by their chemical nature. Our observations, deliberately limited in design to known biologic antigens or nonantigenic substances, fit a general pattern of correlation between antigenicity and this early, transient polymorphonuclear reaction to foreignness. Nonantigenic materials do not elicit an inflammatory reaction, while rabbits actively immunized generally produce a more severe inflammatory reaction to that antigen. Possibly the finding that human serum which has had complement and similar materials inactivated by heating is less inflammatory than fresh serum, supports an immunologic mechanism, but certainly there may be other interpretations. In particular, heat-labile enzymes could be implicated.

The concept of an association between the so-called nonspecific inflammatory reaction and immunity fits well with a series of observations and hypotheses recently expounded by Boyden.[18] In an extensive review of cellular recognition of foreignness, from single-celled animals to mammals,

Boyden postulates that foreign recognition and inflammation occur only when some type of specific humoral substance is present, presumably factors in the still obscure group of naturally occurring antibodies and opsonins.

This hypothesis—that the inflammatory reaction produced by chemically bland soluble proteins is mediated through some naturally occurring antibody-like material—deserves further investigation. For example, an occasional rabbit fails to produce antibodies to a given antigen. Does he also fail to respond by local inflammation to that material? Specific immune tolerance can be produced by inundating newborn rabbits with antigen.[19] Do such rabbits retain their local polymorphonuclear reaction to the antigen? Studies in such experimental models should help to clarify the relationship between the acute inflammatory response to foreign materials, and their antigenicity.

SUMMARY

The acute polymorphonuclear exudation that characterizes reaction to the injection of foreign soluble reactions was studied in the rabbit suprapatellar bursa. Only whole heterologous sera, or materials derived from biologic sources were employed, in order to minimize inflammation due to the chemical nature of the materials. Fresh human sera caused greater exudation than sera heated to 56° C. for 30 minutes. Materials of known antigenicity in the rabbit (human serum albumin and globulin, ovalbumin, bovine serum albumin) caused distinct inflammatory reactions; materials of minimal antigenicity (human hemoglobin, dextran, gelatin) produced little reaction. Immune animals responded with greater synovial reaction than normal animals, but the sequence of exudation was similar. Iodine-^{131} used to label an antigen did not disappear from the synovial area at different rates in normal and immune animals.

These observations, although limited in scope, suggest that the polymorphonuclear reaction to soluble proteins and polysaccharides is related to the immunologic reaction to these materials, rather than that the two phenomena represent different host reactions to foreignness.

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REFERENCES

1. Metchnikoff, Elie: *Leçons sur la pathologie comparée de l'inflammation.* Paris, Masson, 1892.
2. More, R. H. and Movat, H. Z.: Cellular and intercellular changes in the Arthus phenomenon. *Arch. Path.,* 1959, *67,* 679-693.

3. Clark, E. R., Clark, E. L., and Rex, R. O.: Observations on polymorphonuclear leukocytes in the living animal. *Amer. J. Anat.*, 1936, *59*, 123-173.
4. Hollingsworth, J. W. and Atkins, Elisha: Synovial inflammatory response to bacterial endotoxin. In preparation.
5. Ratnoff, O. D. and Lepow, I. H.: Complement as a mediator of inflammation. Enhancement of vascular permeability by purified human C'1 esterase. *J. exp. Med.*, 1963, *118*, 681-697.
6. Kabat, E. A. and Meyer, M. M.: *Experimental Immunochemistry*, Second Edition, Springfield, Illinois, Charles C. Thomas Co., 1961, p. 22.
7. Speirs, R. S. and Speirs, E. E.: Cellular localization of radioactive antigen in immunized and nonimmunized mice. *J. Immunol.*, 1963, *90*, 561-575.
8. Boyden, Stephen: The chemotactic effect of mixtures of antibody and antigen on polymorphonuclear leucocytes. *J. exp. Med.*, 1962, *115*, 453-466.
9. Patterson, Roy, Weigle, W. O., and Dixon, F. J.: Elimination of circulating serum protein antigens as a sensitive measure of antibody. *Proc. Soc. exp. Biol. (N. Y.)*, 1960, *105*, 330-337.
10. Goldberg, B., Kantor, F. S., and Benacerraf, B.: An electron microscopic study of delayed sensitivity to ferritin in guinea-pigs. *Brit. J. exp. Path.*, 1962, *43*, 621-626.
11. Hamerman, David and Schubert, Maxwell: Diarthrodial joints, an essay. *Amer. J. Med.*, 1962, *33*, 555-590.
12. Suter, E. R. and Majno, G.: Ultrastructure of the joint capsule in the rat: Presence of two kinds of capillaries. *Nature*, 1964, *202*, 920-921.
13. Speirs, R. S. and Dreisbach, M. E.: Quantitative studies of the cellular responses to antigen injections in normal mice: Technic for determining cells in the peritoneal fluid. *Blood*, 1956, *11*, 44-55.
14. Litt, Mortimer: Studies in experimental eosinophilia. I. Repeated quantitation of peritoneal eosinophilia in guinea pigs by a method of peritoneal lavage. *Blood*, 1960, *16*, 1318-1337.
15. Cochrane, C. G., Weigle, W. O., and Dixon, F. J.: The role of polymorphonuclear leukocytes in the initiation and cessation of the Arthus vasculitis. *J. exp. Med.*, 1959, *110*, 481-494.
16. Chernoff, A. I.: Immunologic aspects of human hemoglobin. In: *Conference On Hemoglobin, May, 1957*. National Research Council, Publication 557. Washington, D. C., National Academy of Sciences, 1958.
17. Fruhman, G. J.: Extravascular mobilization of neutrophils. *Ann. N. Y. Acad. Sci., 1964, 113*, 968-1002.
18. Boyden, S.: Cellular recognition of foreign matter. *Int. Rev. exp. Path.*, 1963, *2*, 311.
19. Weigle, W. O.: Termination of acquired immunological tolerance to protein antigens following immunization with altered protein antigens. *J. exp. Med.*, 1962, *116*, 913-928.