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Abstract

Mononuclear cells from rabbit joint fluid were studied after synovitis was induced by various means, including the intra-articular injection of bacterial endotoxin or of aggregated human gamma globulin in normal rabbits, or of HGG in rabbits previously sensitized to this material. The large majority of mononuclear cells in all groups were monocytoid rather lymphocytoid, and these cells were most readily labeled with tritiated thymidine on the first day after injection. On day 2 and 3, the numbers of labeled cells decreased, except for the animals previously sensitized with HGG, in which there was an upswing of labeling on day 3. This upswing was associated with a considerable increase in numbers of cells resembing synovial cells, and may possibly be a reflection of synovial proliferation. Macrophages loaded with engulfed polymorphonuclear cells were observed in all experimental groups, a finding which emphasizes the lack of specificity of this reaction.

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MONONUCLEAR CELLS OF RABBIT SYNOVIAL EFFUSION: MORPHOLOGY AND DNA SYNTHESIS

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Mononuclear cells (MNC's) in synovial effusion of patients with joint diseases consist of lymphocytes and a variety of large phagocytic cells with markedly different morphologic features. In the excellent monograph on "Synovial Fluid Changes in Joint Disease", however, ROPES and BAUER (1) have missed these mononuclear cells.

No specific diagnostic significance has been attributed to any of these cells until PEKIN and his associates reported that macrophages loaded with many ingested neutrophils or cell nuclei were characteristic features of Reiter's syndrome (2). We also extensively reviewed our joint fluid studies and found that these macrophages were also abundantly seen in the joint fluid of patients with gonococcal arthritis, gout, pseudogout, psoriatic arthritis as well as juvenile rheumatoid arthritis (3). An additional finding in these studies was that synthesis of DNA in human joint fluid is by no means confined to lymphocytes. Particularly in the fluid from patients with chronic seropositive and seronegative rheumatoid arthritis, many large mononuclear cells, some presumably derived from the synovial lining, are labeled with a DNA precursor (4).

In the current report, for the purpose of confirming the human studies as described above, experimental synovitis was induced in the rabbit knee joint, both with non-specific stimuli and with an intra-articular Arthus reaction. Similar studies were performed on the mononuclear cells in the rabbit synovial exudates and it was again found that not only the lymphocytes but also many large mononuclear cells were capable of dividing in the inflammatory synovial exudate and that macrophages with engulfed neutrophils were frequently encountered in all kinds of synovial effusions elicited with three different sorts of inflammatory stimuli, thus verifying our previous suggestion that these macrophages would have rather limited diagnostic significance, not quite specific for Reiter's syndrome.

MATERIALS

Animals: New Zealand white rabbits of both sexes, weighing 2—3 kg., were used in all experiments. They were fed on regular purina rabbit chow and water was given *ad lib.*

Endotoxin: Lyophilized highly purified bacterial endotoxin derived from *Proteus vulgaris* ("E"-Pyrogen) was obtained from Organon Laboratories, London, England. It was dissolved in sterile pyrogen-free normal saline immediately before use.

Human gamma globulin: Human Gamma Globulin (HGG, Cohn Fraction II) was purchased from Lederle Laboratory, American Cyanamid Corp., Pearl River, New York. Heat-aggregated HGG was prepared by incubating HGG in 5% glycine saline at 63°C for fifteen minutes (5).

H^3-Thymidine: Thymidine-methyl-H^3 with specific activity of 6.7 c/mM was obtained from New England Nuclear Corp., Boston, Mass.

EXPERIMENTAL DESIGN AND PROCEDURES

One-half milliliter of the materials to be studied was injected into the suprapatellar bursae of each rabbit anesthetized with approximately 60 mg of pentobarbital administered intravenously. Fifty-four rabbits were divided into three groups of eighteen each and both knees were used in each case.

The knees of 18 normal rabbits of Group I were injected with 0.0005 μg of bacterial endotoxin. The joints of the second normal group (Group II) were entered with 10 mg of heat-aggregated HGG dissolved in sterile 5% glycine saline. The last group of 18 rabbits (Group III) were presensitized to HGG by giving subcutaneous injection of 40 mg HGG with complete Freund's adjuvant (Difco Laboratory, Detroit, Michigan). The same procedure was repeated two weeks later. Three weeks after the secondary immunization, serum from each animal was collected and microcapillary precipitin test (6) was carried out to detect precipitin antibody. All but one turned out to be positive. Seventeen precipitin-positive rabbits were then challenged by intra-articular injection of heat-aggregated HGG (1 mg) dissolved in 5% glycine saline in order to induce an intra-articular Arthus reaction.

Six rabbits from each group were sacrificed on day 1, 2 and 3 after the inflammatory challenges (Group III had only 5 rabbits sacrificed on day 3). The resulting twelve joints from each group were entered with #19 gauge needle attached to 2 ml. plastic syringe filled with normal saline and 10 units of preservative-free heparin (Fellows-

Testagar, Detroit, Michigan). The suprapatellar bursae were aspirated and then washed with saline by distending back and forth in an attempt to collect as many exudate cells in the joint cavity as possible.

The final volume of exudate-saline was measured, white cells counted by a Model A Coulter electronic particle counter, and differential counts done on the Wright-Giemsa stained smears. The fluid from each joint was then incubated with approximately 2 μc of H^3-thymidine/ml of aspirated joint fluid for 90 minutes in Eberbach shaker. Subsequently the cells were spun down at 1500 rpm in International Clinical Centrifuge Model PR2 for ten minutes, the supernatant discarded, the pellet washed again two times with normal saline by centrifugation and the final pellet was mixed well with one large drop of 30 % bovine serum albumin. By using a fine-tipped sabel brush, the cells were smeared on the alcohol-acetone cleansed slide glass, fixed in the absolute methanol for fifteen minutes and dipped in the NTB emulsion for radioautography (Eastman Kodak Company, Rochester, New York). After two week-exposure in the dark at 4°C, the radioautographs were developed and stained with Wright-Giemsa. Slides were examined under oil immersion microscopy and three thousand mononuclear cells were counted from each joint fluid. Numbers and characteristics of the labeled cells were recorded. The labeling indices were expressed as the numbers of labeled cells per 1, 000 mononuclear cells.

RESULTS

Fig. 1 summarizes the type of cellular responses induced by the different kinds of stimuli on each of the three consecutive days. In Groups I and II on day 1, the exudate cells were predominantly polymorphonuclear. On day 2, however, as the total number of exudate cells decreased, the mononuclear cells outnumbered the polymorphonuclear cells (PMN's). In Group II on day 3, inflammatory reactions rapidly subsided. Consequently the yield of the harvested cells were so low that the valid tabulation of the MNC's and the PMN's was not feasible. In contrast, Group III or the HGG-sensitized group showed marked inflammatory response on day 1, characterized by absolute increase in neutrophils. Eosinophils were also seen. MNC's did not predominate until day 3 in this group.

In all the groups, lymphocytes were few: they rarely exceeded 5 % of the total MNC count, except in Group III, HGG-sensitized group, where they reached 10% on day 2 only.

Also in each group on day 1, when the inflammatory reactions pro-

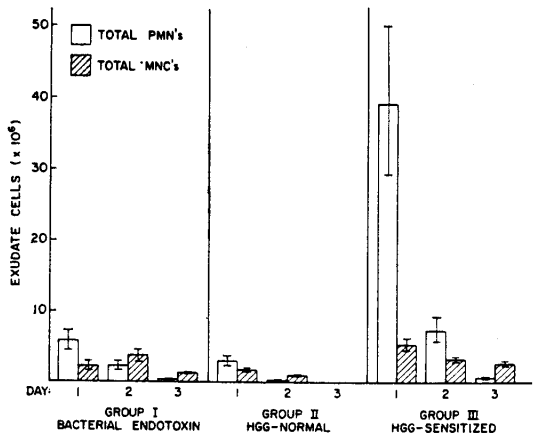

Fig. 1 Number of exudate cells in the rabbit synovial effusion produced by three different kinds of stimuli. The clear areas represent PMN's, and the hatched ones total MNC's.

voked were most severe, fairly a large number of mononuclear cells, most of which resembled active macrophages rather than "blood" monocytes, were observed. An exact differentiation among them, however, was almost impossible because of the many transitional types of cells that were present (Fig. 2). In Groups I and II, as the acute inflammatory responses subsided, large monocytes began to appear in the effusions and typical macrophages got fewer in number. In Group III, however, the morphologic type of the mononuclear cells seemed to undergo a rather notable evolution around this period. On day 3 in this particular group, more than 40% of the total MNC's resembled synovial cells or more accurately, synovial lining cells, with typical eccentric nuclei and large cytoplasm (Fig. 3). They were sometimes multinucleated (Fig. 4). The numerous vacuoles were still seen in most of these cells. It was quite easy, however, to differentiate them from typical plasmacytes which were seen only occationally in the fluid of this group. The synovial lining cells were never observed to that degree in any synovial effusion of the first two groups.

Macrophages with engulfed PMN's were encountered in all three experimental groups. Although getting fewer as the inflammatory responses regressed, they were seen throughout the course. They were abundantly seen even in Group II on day 1. Fragments of pseudoeosinophilic granules of the rabbit PMN's were also frequently observed in the phagocytic vacuoles of these macrophages.

Fig. 5 shows the number of DNA synthesizing cells in the joint fluid

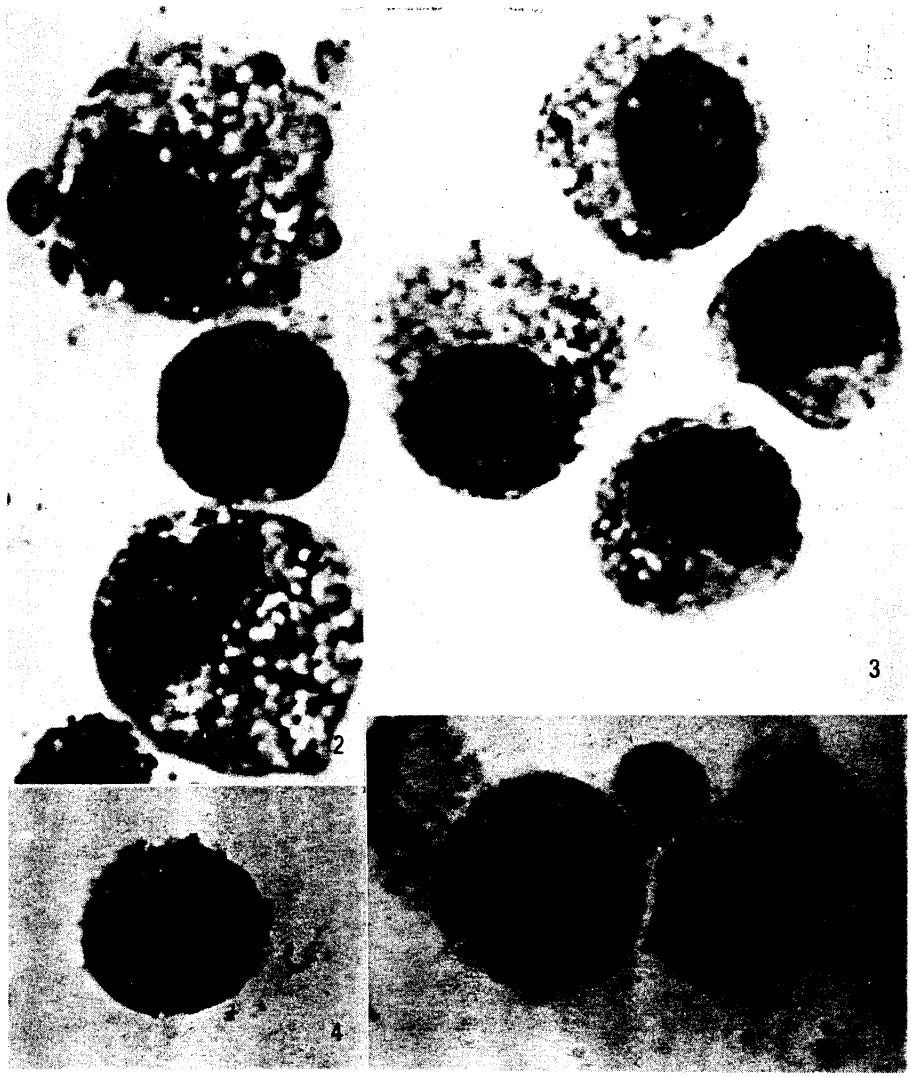

Fig. 2 Large mononuclear cells observed at the beginning of the synovitis induced with intra-articular Arthus reaction in Group III. They are mostly macrophages and monocytoid cells.

Fig. 3 Large mononuclear cells with eccentric nuclei and large cytoplasm, presumably deriving from the thickened synovia, comprised more than 40% of the total MNC's on day 3 in the hypersensitized group. Please notice the close resemblance between these and those cells obtained from the normal synovial lining in the normal rabbit knee joint as shown in Fig. 8.

Fig. 4 One large binucleated cell in the synovial fluid (Group III, day 3).

Fig. 6 Two labeled large mononuclear cells and one small lymphocyte which is unlabeled are seen. (Group III, day 3). Sometimes grains are too heavy to permit accurate differential between the lymphocytoid and the monocytoid one.

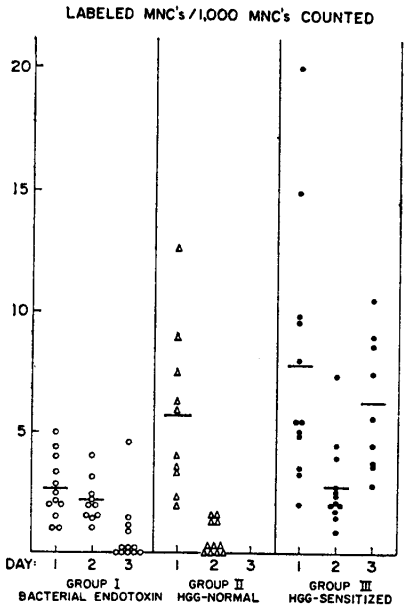

Fig. 5 Number of labeled cells per 1,000 mononuclear cells in the synovial fluid in each group.

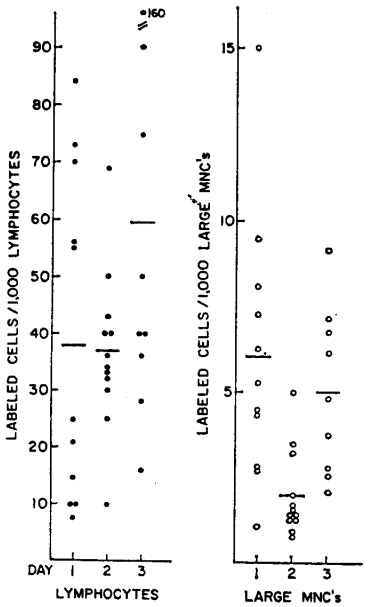

Fig. 7 Labeling of lymphocytes (per thousand) and large mononuclear cells (per thousand) in joint effusions obtained from the hypersensitized group on three consecutive days.

from each group. In Groups I and II of this figure, the number of the labeled cells diminished concomitantly along with rapid subsidence of inflammatory reactions in Fig. 1. The labeling index of the Group II on day 1 was rather high, compared to that of Group I on the same day and on day 2 it dropped very precipitously to zero point.

On the other hand, in the hypersensitized group, the numbers of labeled cells decreased on day 2 and then increased on day 3. The changes in labeling indices during three successive days in this particular group are significant with p values of less than 0.01. Only in this hyperimmunized group, the labeled mononuclear cells were differentially counted between lymphoid cells and larger mononuclear cells in an attempt to determine the reproductive capability of each type of cells, although it was sometimes difficult to determine the characters of those cells of which nuclei were frequently heavily covered with silver grains (Fig. 6). As shown in Fig. 7, the labeling indices of the lymphocytes tended to distribute in wider range and these variations of the indices were not statistically significant. On the contrary, the daily change in the labeling

Fig. 8 Normal synovial lining cells obtained by trypsinization of the knee joint cavity in the normal rabbit. They are stained with Wright-Giemsa.

indices of large mononuclear cells showed the same pattern in those of the whole labeled MNC's, as compared in Fig. 5 and Fig. 7.

Fig. 8 shows the normal rabbit synovial lining cells obtained by trypsinizing the joint cavity of the normal rabbit knee according to the method reported by FRASER (7) and WILLIAMSON *et al.* (8). A striking similarity between these and those large MNC's shown in Fig. 3 is quite evident.

From these two points one can say that the "upswing" of labeling index on day 3 in the hypersensitized group is primarily due to the presence of newly appearing large mononuclear cells capable of synthesizing DNA and that they may be probably derived from the thickened synovium *per se*.

DISCUSSION

This study on mononuclear cells of the rabbit synovial effusion was undertaken to confirm and extend our previous studies performed on the MNC's derived from the human synovial exudate. In the human studies, first of all, we discovered that the macrophage studded with engulfed neutrophils had rather limited significance in diagnosing REITER's syndrome: they were abundantly seen in the joint fluids from the patients with a variety of joint diseases. Secondly, we found that cells capable of dividing in the synovial effusions were by no means exclusively lympho-

cytes. Many large mononuclear cells were actively incorporating H^3-thymidine, a DNA precursor.

In order to determine if these human findings would be quite applicable to the exudative MNC's in the rabbit synovial effusion, acute inflammatory reactions were elicited in the rabbit knee joint by using three different kinds of stimuli that were available. Purified bacterial endotoxin and human gamma globulin were selected as non-specific stimuli in the normal rabbits. For inducing antigen-antibody mediated synovial inflammation, an Arthus type of reaction was provoked in the synovial cavity of the rabbit presensitized to HGG, by introducing heat-aggregated HGG intra-articularly.

The inflammatory response elicited by intra-synovial injections of bacterial endotoxin has been extensively studied by HOLLINGSWORTH *et al.* (9). They were mainly concerned with the neutrophilic exudate. They used 0.0005 μg of the proteus endotoxin, the same dose as used in this study, and followed the whole course up to two days after injection: at 24 hours after injection the mean exudate was about 11 million cells with 95% PMN's, and on the second day total WBC dropped to 0.9 million cells (12% PMN's) with almost 100% large monocytes present. In the present case, the synovial inflammations were slightly more protracted. Even on day 3 (which incidentally they had not studied so far), the mean exudate for PMN's and MNC's were 0.17 million and 1.15 millions respectively. However, in general, the current study agreed with and reconfirmed their findings on the synovial inflammation induced with a single injection of the bacterial endotoxin.

At the beginning of the synovitis, MNC's were mostly macrophages with large phagocytic vacuoles which frequently contained pseudoeosinophilic granules of the rabbit PMN's or PMN's themselves. As the inflammatory response diminished, blood monocytoid cells, though a little larger than the typical one seen in the peripheral blood, took over and throughout the course synovial lining cells which are mentioned earlier, were quite rarely seen. LEWIS and his colleague also investigated the histology of the endotoxin-induced synovitis and found that the inflammatory reaction elicited with single injection of the substance was quite brisk and transient and that it caused temporary derangement and hypertrophy of the cells lining the synovial membrane to a limited degree (10). This would partially explain the minimal response of the MNC's in the later stage in the synovial effusions of this group.

DNA synthesizing cells were studied for the first time in this particular group of the synovial effusion, by incubating the cells *in vitro* with a DNA

precursor after joint fluid was harvested. On day 1, the labeling indices were fairly low (2.6 (mean)$\pm$1.3(1 S. D.)/1, 000 MNC's counted) and most of the labeled cells looked like large MNC's. Lymphocytes, though rare in number, were also labeled occasionally. With the inflammatory reactions subsiding, the indices gradually decreased and on day 3 they reached almost zero point, suggesting that the monocytoid cell seen in the later stage of the inflammation, was a "well-developed" end cell, acting primarily as a local scavenger.

HOLLINGWORTH also extensively worked on cellular reactions to soluble foreign materials by inducing experimental synovitis in the rabbit knee joint with these substances. Whole human serum, human serum albumin and globulin, ovoalbumin and bovine serum albumin were used in his study. In all, he could elicit the distinctive inflammatory response, more transient in nature than the endotoxin group, with MNC's predominating at 48 hours after challenge (11). Human gamma globulin was chosen in this study as the soluble foreign material, mainly because this Group II was selected as serving the control for the next hyperimmunized group to this material. It can be said that HGG could cause definite inflammatory reactions, well reflected on the number and types of cells that were seen in the earlier stage after the intra-articular challenge: at the beginning the macrophages were predominant, though most of the labeled cells were monocytoid and the lymphocytes were very rarely seen. The large macrophages were replaced, in much faster speed than in Group I, with smaller monocytoid cells which were not labeled, again suggesting that the synovial derangement induced with the foreign material was quite minimal and the repairing process of the synovia was accomplished within two days after the inflammatory stimulus was introduced.

In contrast to the above two groups, the third group, where intra-articular Arthus reaction was induced by HGG, the inflammatory response was far more pronounced: the initial reaction was characterized with absolute increase in PMN's exudate and the presence of numerous large phagocytic macrophages with engulfed PMN's. Lymphocytes were few even in this hypersensitized group (This is in keeping with observation that the classical Arthus reaction in rabbits is a predominantly polymorphonuclear inflammatory reaction (12)). In some, the labeling indices were extremely high with mostly monocytoid cells labeled. On day 2, with the inflammatory reactions subsiding, just as seen in Group I and II, PMN's decreased in number and many transitional forms of cells between monocytoid and large macrophages were observed with the labeling indices declining. Most interesting phenomenon, thus far reported

nowhere, was then discovered on day 3: large MNC's which we take to have derived from the synovial lining appeared in the synovial effusion, many of which were actively incorporating a DNA precursor as seen in Fig. 6 and these synovial lining cells comprised more than 40% of the total MNC's. The labeling indices were elevated.

One would be struck with the paucity of the literature concerning the synovial cells (or synovial lining cells) in their exfoliative forms. FRAZER originally described a method of stripping off the lining cells from the synovia of the knee joints at autopsy by the use of trypsin (7). WILLIAMSON and his associates (8), following FRAZER's original, obtained the cells, stained with Jenner-Giemsa and delineated two types of cells:

(i) Numerous type I cells containing pale blue-grey cytoplasm with numerous azurophilic granules, oval shaped nucleus situated mainly in a central position with one of two prominent nucleoli present.

(ii) Less frequent type 2 cells containing a large eccentric, darkly-staining nucleus and deeply basophilic cytoplasm.

No attempt thus far to type out the normal rabbit synovial cells has been undertaken. By analogy, however, most of the normal synovial lining cells obtained from the normal rabbit knee joint by trypsinization, do look like human type 2 cells with large ecentric nuclei (Fig. 8). To further delineate the types of large MNC's for which we presumed to have derived from the synovial lining on day 3 in the HGG-hypersensitized group, is quite difficult, for they were more or less activated and tranformed by the smoldering inflammatory stimuli. Yet in Fig. 3, some looked like human type 1 cells and others quite resembled type 2 cells derived from the normal human synovial lining.

Numerous ultrastructural studies were also performed on the synovial membrane derived from normal individuals (13), from patients with rheumatoid arthritis (14, 15, 16), from osteoarthritic patients (17), and so forth. BARLAND and HAMERMAN (13), in their study on normal synovial lining cells from human beings, divided them into two types or states of activity on the basis of their cytoplasmic contents:

(i) Predominant type A cells rich in lysosomes and pinocytic processes, possessing numerous large vacuoles and primarily engaged in active phagocytosis.

(ii) Less frequent type B cells containing abundant ergastoplasm, less extensive Golgi apparatus and few vacuoles, mainly engaged in synthetic work.

However, intermediate forms which could not be placed in either group were frequently encountered. NORTON (15) then suggested that

Type A and Type B cells represent different but probably interchangeable functional states of the same connective tissue cell type. WILLIAMSON *et al.* (8) further tried to reconcile their classification of the normal human synovial cells with that of BARLAND and HAMERMAN, just described, and they presumed that their predominant Type 1 cell corresponded to the predominant Type A cell of BARLAND and HAMERMAN, and similarly the Type 2 cell corresponded to the Type B cell.

Rabbit synovial membrane was extensively studied by GHADIALLY and ROY (18) and just as in the human studies, two types of cells were also noted in the rabbit synovia, with many intermediate forms showing features of both types present.

Following the intra-articular injection of a variety of small particulate substances like gold (19), thorotrast (20), iron dextran (21) and carbon (22), it has been shown that they were readily taken up by synovial lining cells and cells underlining the membrane, and GHADIALLY and ROY (23) again showed in the ultrastructural study of rheumatoid synovium that these cells could incorporate larger particulate substances such as red blood cells.

Once one takes into acconnt these well-established findings, it may well be postulated that the mononuclear cells of many transitional forms and even typical macrophage-like cells with many phagocytic vacuoles, seen in the joint fluid harvested from the hyperimmunized rabbits, could be solely derived from the synovial lining cells. At the onset of the severe inflammation they could take more active forms, namely, macrophages and engage themselves in very active phagocytosis and finally be detached from the lining. Later with the subsidence of the inflammatory reaction and with the repairing processes taking over, rapidly proliferating synovial cells of typical form could be shed off from the presumably thickend synovium. The origin and DNA synthesis of these cells in the synovial membrane *per se* in the hypersensitized group will be the subject of the next study.

SUMMARY

Mononuclear cells from rabbit joint fluid were studied after synovitis was induced by various means, including the intra-articular injection of bacterial endotoxin or of aggregated human gamma globulin in normal rabbits, or of HGG in rabbits previously sensitized to this material. The large majority of mononuclear cells in all groups were monocytoid rather lymphocytoid, and these cells were most readily labeled with tritiated

thymidine on the first day after injection. On day 2 and 3, the numbers of labeled cells decreased, except for the animals previously sensitized with HGG, in which there was an upswing of labeling on day 3. This upswing was associated with a considerable increase in numbers of cells resembing synovial cells, and may possibly be a reflection of synovial proliferation.

Macrophages loaded with engulfed polymorphonuclear cells were observed in all experimental groups, a finding which emphasizes the lack of specificity of this reaction.

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