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11. Blood was routinely drawn from a vein in the arm with disposable plastic syringes. Platelet-rich plasma was obtained by centrifugation of 40 ml of blood plus 4 ml of 3.2 percent trisodium citrate in a plastic tube at 4°C for 10 minutes at 500 rev/min (130*g*). Usually 12 to 15 ml of PRP was pipetted off and the remaining plasma and blood were centrifuged for 15 minutes at 5000 rev/min (1500*g*) at 4°C to secure platelet-poor plasma. The plasmas were kept in an ice bath at 4°C and tested as soon as possible.
12. The concentration of 2,3-DPG used to demonstrate its inhibitory effect on aggregation (20 to 200 $\mu M$) is approximately 5 to 50 times greater than that detected in erythrocytes (*3*). However, in some experiments we have observed a significant inhibitory effect with 5 to 20 $\mu M$ 2,3-DPG. Furthermore, local concentrations of 2,3-DPG released from erythrocytes in vivo and closely adjacent to the platelets may be considerably higher than those used in our in vitro experiments. In addition, in anemias and hypoxias 2,3-DPG concentrations are significantly increased (*7*).
13. Platelet-rich plasma was placed in the aggregometer; either isotonic saline or 2,3-DPG plus ADP, epinephrine, or norepinephrine were added; and the solutions were incubated for 0, 2, or 5 minutes. After the incubations, 0.1-ml portions were removed and pipetted into test tubes containing 0.1 ml of 25 m$M$ calcium chloride and 0.1 ml of a solution consisting of 10 $\mu$g of Russell viper venom (Stypven of Burroughs Wellcome) per milliliter of imidazole buffered saline. The clotting times of these mixtures were measured and the results were expressed (Fig. 2) as the percentage decrease of the Russell viper venom time of the samples incubated for 2 and 5 minutes compared to the sample incubated for 0 minute [M. B. Zucker and J. Peterson, *Proc. Soc. Exp. Biol. Med.* **127**, 547 (1968)]. In Table 1 the results are expressed as the percentage changes of the reciprocals of the clotting times for the 2- and 5-minute samples compared with the corresponding 0-minute sample.
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## Collagenase Production by Lymphokine-Activated Macrophages

Abstract. *Macrophages incubated with products (lymphokines) secreted by stimulated spleen cells produced collagenase. Active lymphokines were obtained both from mitogen- and antigen-stimulated lymphocytes. These observations suggest that the degradation of collagen in chronic inflammatory lesions may be caused by macrophage collagenase.*

Inflammatory lesions characterized by an infiltrate of mononuclear cells are often destructive to the surrounding connective tissue. The predominance of lymphocytes and macrophages in such a lesion suggests that they may have a central role in the degradation of connective tissue. Macrophages of alveolar origin have been shown to contain collagenase (*1*). However, macrophages from peritoneal exudates lack collagenase, but will secrete the enzyme when activated in culture by endotoxin (*2*). Since activated lymphocyte products are capable of stimulating certain functions of macrophages (*3*), we have tested the ability of soluble products (lymphokines) produced by mitogen- or antigen-activated lymphocytes to induce macrophages to produce collagenase.

Macrophages were obtained from the peritoneal cavity of guinea pigs 4 days after the injection of 20 ml of sterile mineral oil (Drakeol, Pennsylvania Refining). Dulbecco Vogt's medium (10 ml; NIH) containing $4 \times 10^6$ to $5 \times 10^6$ washed cells per milliliter was placed in plastic culture flasks (75 cm$^2$; Falcon). After incubation for 4 hours at 37°C, the nonadherent cells were removed by several washings with media. The remaining cells were 94 to 96 percent macrophages, as judged by their appearance and by the ability of cells to phagocytize latex particles, and 4 to 6 percent lymphocytes.

Soluble products produced by activated lymphoid cells were obtained as follows. Guinea pigs were immunized with 50 $\mu$g of dinitrophenylated ovalbumin (DNP-OA) in complete Freund's adjuvant by injection in the footpads. Two weeks later the spleens were excised from these animals, and cultures of splenocytes were prepared (*4*). The lymphoid cells ($5 \times 10^6$ cells per milliliter) were suspended in serum-free RPMI 1640 medium (Grand Island Biological) or Dulbecco Vogt's medium and cultured in the presence or absence of DNP-OA (10 $\mu$g/ml), DNP–bovine serum albumin (10 $\mu$g/ml), or concanavalin A (1 $\mu$g/ml) (Calbiochem). After 24 hours, the cells were removed from the media by centrifugation, and the supernatants were dialyzed against distilled water and lyophilized. Antigen or mitogen was added to nonstimulated spleen cell cultures just prior to centrifugation, which served as a control. These products from the lymphoid cell cultures were added to the macrophage cultures at a concentration of 50 $\mu$g/ml. Media from the macrophage cultures were harvested daily and dialyzed against 0.03 m$M$ tris ($p$H 7.5) and lyophilized.

The media (20 ml) in which macrophages were cultured were concentrated and assayed for collagenase with reconstituted collagen fibrils biosynthetically labeled with [$^{14}$C]glycine (*5*). Under physiological conditions cleavage of the helical portion of the collagen molecule is limited to collagenase. Trypsin (0.01 percent) was added to certain collagen substrates to obtain a measure of the substrate that could be solubilized without cleavage of the helical portion of the molecule. The reaction mixtures were incubated at 35°C for 16 hours, and the collagenase activity was then determined by measuring the labeled material in the supernatant.

Since we have observed that peritoneal macrophages produce collagenase after incubation with endotoxin (*2*), it was of interest to determine whether products from activated lymphocytes which stimulate various macrophage functions could also enhance collagenase synthesis. Indeed, when products from spleen cells stimulated with concanavalin A were added to macrophage cultures, significant collagenase activity was detected within 48 hours (Fig. 1). No collagenase activity was detected in media obtained from stimulated or control spleen cells. When the products produced by concanavalin A–stimulated cultures were added to macrophages in culture, collagenase activity was increased after a 1-day lag period. Essentially all the collagenase activity was present in the media. No increase in activity was observed in macrophage cultures receiving products from unstimulated spleen cells. In addition, products from supernatants of control spleen cells to which concanavalin A was added at the termination of culture did not induce collagenase release by the macrophages (Fig. 1), nor did they contain collagenase activity. In contrast to earlier studies in which lymphokine enhancement of macrophage function was not significant for 3 days (*3*), macrophage collagenase activity was maximum at 48 hours.

Table 1. Antigen-induced lymphocyte product activation of macrophages to produce collagenase. Spleen cells from guinea pigs immunized to dinitrophenylated ovalbumin (DNP-OA) in complete Freund's adjuvant were cultured for 24 hours with DNP-OA, DNP–bovine serum albumin (DNP-BSA), or purified protein derivative (PPD) (10 μg/ml) or in media only. Nonstimulated lymphocyte supernatants were reconstituted with equivalent amounts of the antigens at the end of culture. Concentrated lymphocyte supernatants (50 μg/ml) were then added to macrophages obtained from nonimmune animals. Concentrated media from the macrophage cultures were assayed on day 2 for collagenase activity on [$^{14}$C]glycine-labeled collagen substrates. Collagenase activity is represented as the number of counts per minute of radioactivity released into the supernatant from the substrate (1150 count/min per substrate). The trypsin control released 110 count/min.

| Lymphocyte stimulant | Macrophage collagenase activity (count/min) |
|---|---|
| DNP-OA | 817 |
| DNP-OA reconstituted | 88 |
| DNP-BSA | 55 |
| DNP-BSA reconstituted | 93 |
| PPD | 960 |
| PPD reconstituted | 89 |
| None | 28 |

Since lymphokines are also produced in vitro when lymphocytes are exposed to antigens to which they are sensitive (*3*, *4*), we tested such products for their ability to increase collagenase levels in macrophage cultures. When spleen cells from animals immunized with DNP-OA were stimulated in vitro with DNP-OA, they secreted a material that induced macrophages from nonsensitized animals to produce collagenase (Table 1). Production of this inducer was antigen specific since DNP–bovine serum albumin would not substitute for DNP-OA (Table 1). Purified protein derivative also caused lymphocytes from specifically immunized animals to release soluble factors that induced macrophages to produce collagenase (Table 1). Reconstitution of nonstimulated lymphocyte supernatants with equivalent amounts of purified protein derivative before addition to macrophage cultures did not induce these cells to produce collagenase. Thus, it appears that products from antigen- or mitogen-stimulated lymphocytes have the capacity to induce macrophages to synthesize the enzyme collagenase. No collagenase activity was observed after the addition of cycloheximide (1 μg/ml) to macrophages that had been exposed to products from activated lymphocytes, an indication that the increase in collagenase activity was dependent on protein synthesis.

In order to determine the site of cleavage by the macrophage enzyme we precipitated the collagen molecules from a reaction mixture into ordered aggregates or segment-long-spacing (SLS) collagen as follows (*6*). Portions from reaction mixtures incubated at 25°C were dialyzed at 4°C against 0.1*M* acetic acid and then against 0.4 percent adenosine triphosphate in 0.1*M* acetic acid for 24 hours. A drop of the resulting suspension was placed on individual Parlodion-carbon coated copper grids (400 mesh) and stained after drying with 1 percent phosphotungstic acid and subsequently with 1 percent uranyl acetate. The grids were examined in an AEI-6B electron microscope. The enzyme predominantly cleaves the collagen molecule at a site 62 percent of the distance from the amino terminus; this amino terminal portion is shown hybridized to the intact collagen molecule (Fig. 2). While the site of cleavage of the purified collagenase from tadpole tail (*7*), human skin (*8*), and granulocytes (*9*) is 75 percent from the amino terminus, cleavages of 62 or 67 percent have been reported for purified collagenases from the uterus (*10*) and skin (*11*) of rat. Since the macrophage preparations used were impure, an initial cleavage could have occurred 75 percent from the amino end with subsequent reductions by other proteases.

Collagenase has been found in several chronic inflammatory lesions in which the immune response appears to be involved, including rheumatoid arthritis (*12*) and periodontal disease (*13*). Our findings suggest a mechanism for the events leading to the destruction of connective tissue in such chronic inflammatory lesions. Antigen-activated lymphocytes produce several mediators that modulate macrophage function, including a chemotactic factor (*4*, *14*) that may attract large numbers of macrophages to the inflammatory locus. Once at the inflammatory site, lymphokines can inhibit further macrophage migration (*15*) and cause the activation of these cells (*3*). The production of collagenase by these activated macrophages may result in the extracellular breakdown of collagen with subsequent degradation occurring intracellularly aided by the increased acid hydrolases in activated macrophages (*16*).

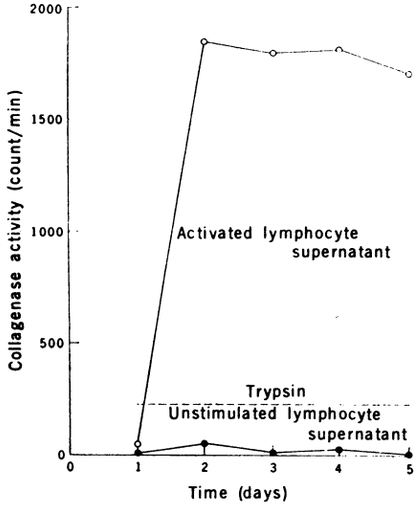
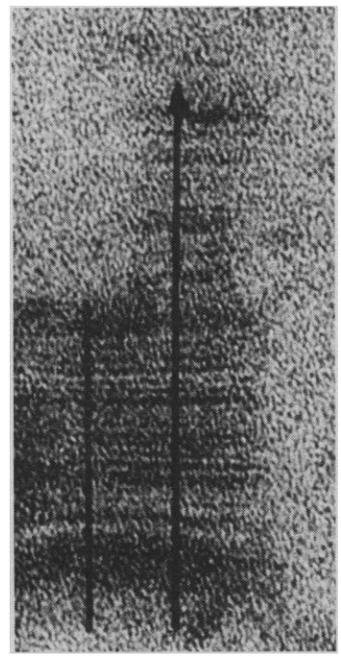



Fig. 1 (left). Increased collagenase production by macrophages receiving products (50 μg/ml) from lymphocytes stimulated with concanavalin A in culture. Collagenase activity was assayed in the media from macrophage cultures with the use of $^{14}$C-labeled collagen fibers (2200 count/min per substrate). Trypsin (0.01 percent) was added to some collagen substrates to estimate the maximum amount of label that could be solubilized by nonspecific proteases. Fig. 2 (right). An electron micrograph of a segment-long-spacing (SLS) crystallite prepared from a reaction mixture of macrophage enzyme and collagen. The long arrow represents the length of intact collagen molecules which are hybridized to collagen molecules cleaved 62 percent of the distance (short line) from the amino terminus (× 300,000).



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## Heavy Cosmic-Ray Exposure of Apollo Astronauts


Abstract. *A comprehensive study of the heavy-particle cosmic-ray exposure received by the individual astronauts during the nine lunar Apollo missions reveals a significant variation in the exposure as a function of shielding and the phase of the solar cycle. The data are useful in planning for future long-range missions and in estimatting the expected biological damage.*


Unlike low-LET (linear energy transfer) radiation (such as $\gamma$ and $\beta$ emissions) which has always been a part of the human environment, heavy-particle, high-LET radiation and weightlessness are two unique aspects of the space environment. Recently, there has been an increasing awareness that high-LET radiation may be considerably more hazardous than the nominal absorbed doses from this type of radiation would indicate. This awareness stems in part from the fact that low-dose, high-LET radiation effects, such as light flashes in humans (*1*) and a variety of other effects in biological systems (*2*), have been observed. However, the body of information and the degree of understanding of these phenomena is meager. Thus, at this time the evaluation of possible long-term effects of such radiation, such as life shortening, carcinogenesis, or a decrease in mental or general performance during long-term missions, cannot be properly assessed and must await the results of future investigations. Although some of these effects may possibly become apparent from the observation of the exposed American and Russian astronauts, a systematic, detailed understanding of the effects of high-LET radiation will have to await the results of accelerator-based studies. Information on these effects will also be important for patients exposed to energetic heavy ions and negative pions during the experimental radiotherapy now in the planning stages at several laboratories in the United States, Europe, and Canada.

During the last several years, using plastic nuclear track detectors, we have developed methods of measuring particle parameter statistics that can be used to compute the integrated quantities of biological significance. These statistics are given in physical terms so that they will remain applicable as the biological effects of the energetic, high-$Z$ ($Z$ is atomic number) particles become better understood (*3–7*).

During the Apollo program, this very high-LET ($LET_{350\,ev} > 130$ kev/$\mu$m in tissue or $LET_{350\,ev} > 150$ kev/$\mu$m in Lexan) (*8*) radiation component was measured with the use of Lexan plastic nuclear track detectors. The Lexan detectors are arranged in packets consisting of three layers, each approximately 8 cm$^2$ in area. Each astronaut wears three packs, one each on the chest, thigh, and ankle. An additional pack is located in the film bag. Because of the small detector area and the relative insensitivity of these detectors, the particles recorded in sufficient numbers to contribute significantly to the counting statistics are those principally from the galactic cosmic-ray beam, with atomic numbers in the range $8 \leq Z \leq 26$ and having an $LET^{\text{Lexan}}_{350\,ev} \geq 150$ kev/$\mu$m. For the most part, the particles recorded are those that stop in or near the detectors (the so-called "enders" or "thin-downs").

Although, in principle, the high-LET

Table 1. Apollo mission data; CM, command module; LM, lunar module; LS, lunar surface.

| Apollo mission* | Type of mission | Launch date | Astronaut | Detector location | Total mission length (hours) | Effective mission length† (days) |
|---|---|---|---|---|---|---|
| 8 | Lunar orbiting | 12-21-68 | F. Borman<br>J. A. Lovell, Jr.<br>W. M. Anders | CM<br>CM<br>CM | 147 | 5.57 |
| 10 | Lunar orbiting | 5-18-69 | T. P. Stafford<br>J. W. Young<br>E. A. Cernan | CM, LM<br>CM<br>CM, LM | 192 | 6.59 |
| 11 | Lunar landing | 7-16-69 | N. A. Armstrong<br>M. Collins<br>E. E. Aldrin, Jr. | CM, LM, LS<br>CM<br>CM, LM, LS | 195 | 6.77 |
| 12 | Lunar landing | 11-14-69 | C. Conrad, Jr.<br>R. F. Gordon<br>A. L. Bean | CM, LM, LS<br>CM<br>CM, LM, LS | 244.5 | 8.20 |
| 13 | Lunar flyby | 4-11-70 | J. A. Lovell, Jr.<br>J. L. Swigert<br>F. W. Haise | CM, LM<br>CM, LM<br>CM, LM | 143 | 5.83 |
| 14 | Lunar landing | 1-31-71 | A. B. Shepard, Jr.<br>S. A. Roosa<br>E. D. Mitchell | CM, LM, LS<br>CM<br>CM, LM, LS | 216 | 7.52 |
| 15 | Lunar landing | 7-26-71 | D. R. Scott<br>A. M. Worden<br>J. B. Irwin | CM, LM, LS<br>CM<br>CM, LM, LS | 295.2 | 9.14 |
| 16 | Lunar landing | 4-16-72 | J. W. Young<br>T. K. Mattingly II<br>C. M. Duke | CM, LM, LS<br>CM<br>CM, LM, LS | 265.9 | 8.33 |
| 17 | Lunar landing | 12-7-72 | E. A. Cernan<br>R. E. Evans<br>H. H. Schmitt | CM, LM, LS<br>CM<br>CM, LM, LS | 301.5 | 9.33 |

* Apollo 9 was strictly an earth orbital mission and was not included in this study. ‡ Transluna time plus one-half the lunar orbit plus the transearth time.