PYROGEN RELEASE FROM HUMAN SYNOVIAL EXUDATE CELLS

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BENNETT and Beeson (1953) first reported that an endogenous pyrogen could be isolated from rabbit granulocytes. Subsequent work by Wood and others (Kaiser and Wood, 1962; Berlin and Wood, 1964*a*) has defined many of the characteristics of the reaction in which pyrogen is released from polymorphonuclear leucocytes (PMN) obtained from rabbit peritoneal exudates. Such leucocytes, mobilized in response to intraperitoneal injections of either saline or glycogen, actively release pyrogen when incubated in saline for a number of hr. Leucocytes obtained from blood, on the other hand, do not release pyrogen under these conditions, but require some stimulus for " activation ". These stimuli include endotoxin, some viruses, antigen-antibody reactions such as tuberculin in sensitized tissues, and phagocytosis of particles or bacteria (Atkins and Snell, 1965). The " spontaneously " active state of PMNs obtained from exudates appears to be due to the prior action of a macromolecular substance normally found in such exudate fluids (Wood, personal communication).

Recently, endotoxin and phagocytosis of heat-killed staphylococci have been shown to activate human blood leucocytes *in vitro* to release a pyrogen that produces fever in rabbits (Bodel and Atkins, 1966). The demonstration that human leucocyte pyrogen can be tested in rabbits made it practical to look for sites of release of endogenous pyrogen in human disease. Granulocytes in areas of inflammation appear to be likely sources of such pyrogen. Since joint fluid from various types of inflammatory arthritis contains many granulocytes, human exudate cells from these sites were studied to determine whether release of pyrogen occurred " spontaneously ", or after *in vitro* activation. Fluid from inflamed joints was also tested for pyrogenic activity.

MATERIALS AND METHODS

Heparinized blood and joint fluid (10 units of heparin per ml. of fluid) were obtained from patients with joint disease. In most instances both fluids were obtained on the same occasion from the same patient. Joint fluid was filtered through several layers of sterile gauze, after which the white cells were centrifuged at 2000 r.p.m. (Int. Cent. No. 2) at 15° for 20 min. The supernatant was removed, and later centrifuged at 3000 r.p.m. at 4° for 30 min. The white cells were suspended in about 30 ml. of heparinized Krebs-Ringer phosphate (KRP) buffer (containing $\frac{1}{5}$ the concentration of calcium and magnesium to prevent precipitation) and centrifuged at 1500 r.p.m. for 15 min. Blood was allowed to sediment, the plasma was removed and centrifuged, and the cells then washed once with buffer as described for cells from joint fluid. After the final centrifugation, both types of cells were suspended in a small volume of buffer, and white counts were performed with a Coulter particle counter. Differentials were done on all joint fluid leucocyte preparations.

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Incubation at 37° was carried out in 50 ml. centrifuge tubes in a stationary incubator, or in 50 ml. Erlenmeyer flasks placed for the first few hr. on a Dubnoff shaker. Flasks were used for all experiments in which phagocytosis was required. The times of incubation and specific media employed varied somewhat and are described in individual experiments. The cell numbers were usually 5–20 $\times$ 10^7 per flask, in a total volume of 10–15 ml. Glucose, 1·5 mg./ml., was usually added for all overnight incubations. When bacteria were used, heat-killed *Staphylococcus albus* was prepared as described previously (Bodel and Atkins, 1966).

After incubation, the contents of the flasks were centrifuged at 1500 r.p.m. for 15 min., or 3000 r.p.m. for 30 min. in experiments in which bacteria were used. A volume of supernatant derived from a chosen number of white cells was injected intravenously into 1 or several recipient rabbits. Usually 2 rabbits received duplicate injections, and the fever curves were subsequently averaged. Joint fluid and plasma supernatants were injected in volumes of 7–13 ml.; sufficient material was generally available for only 1 injection. Pyrogen assays were performed in rabbits by standard procedures used in our laboratories (Atkins and Heijn, 1965). Precautions for keeping glassware and solutions pyrogen-free have been described previously (Atkins and Heijn, 1965).

Joint fluids were obtained from patients with several different types of inflammatory synovitis, although most of the patients studied had rheumatoid arthritis.

RESULTS

Initial studies were carried out to determine whether PMN from joint fluid would release pyrogen spontaneously when suspended in saline. Leucocytes were washed once, suspended in heparinised saline, sometimes with glucose, and incubated for 18 hr. Supernatants were then injected into rabbits. In 13 experiments, in which joint fluids from 11 different patients were tested, release of pyrogen could not be demonstrated. A typical experiment is shown in Fig. 1, lower line. Joint

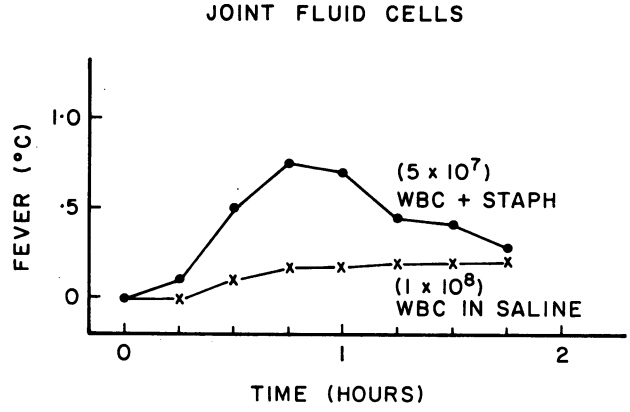

FIG. 1.—Average fevers in 2 rabbits after injection of supernatants from joint fluid cells obtained from a patient with rheumatoid arthritis. Cells were incubated in saline (WBC in saline) or in buffered cell-free joint fluid with heat-killed *Staph. aureus* (WBC + *Staph. aureus*). The numbers of cells from which supernatant was derived for each injection are shown in parentheses.

fluid cells, obtained from a patient with rheumatoid arthritis, were incubated for 18 hr. in saline with added heparin (10 u/ml.) and glucose. The differential count was 90 per cent polys, 9 per cent lymphs, 1 per cent monocytes. After incubation, cell-free supernatant fluid from 1 $\times$ 10^8 leucocytes was injected into 2 rabbits, and the temperature responses were averaged for each curve. These supernatants

were not pyrogenic. For comparison, as shown in the upper line, the same cells did release pyrogen when stimulated by phagocytosis.

In the 13 experiments in which cells from joint fluid were incubated in saline, supernatants from numbers of PMN varying from 5–20 $\times$ 10^7 were injected. No fevers of over 0·2° were observed. Most injections were given in duplicate, and the responses of 2 rabbits averaged. Although inactivation of endogenous pyrogen in saline suspensions of white cells has not been reported, it was considered worthwhile to test release of pyrogen after only 4 hr. of incubation. Cells from the same patient used in the experiment shown in Fig. 1 were incubated for only 4 hr. in saline, and supernatant fluid from 8 $\times$ 10^7 cells injected. Again, as with the 18 hr. incubation (lower curve of Fig. 1), no pyrogen release was demonstrated.

Included in these negative responses were joint fluid cells from 2 patients with rheumatoid arthritis who had negative serum tests for rheumatoid factor, 7 patients with sero-positive rheumatoid arthritis, 1 with metastatic tumour to bone, and 1 with pseudogout.

Attempts to demonstrate release of pyrogen were also made with joint fluid cells suspended in a more complex medium, including buffer alone, or plasma or joint fluid supernatant and buffer, all with added glucose. No release of pyrogen was observed in 5 experiments with cells from 4 different patients. On 2 occasions, however, joint fluid cells of 1 patient did release pyrogen when the cells were suspended in plasma and buffer or joint fluid and buffer. Seven–10 $\times$ 10^7 PMNs were required to elicit an average fever of 0·45° in 4 rabbits. This patient had an unusual inflammatory arthritis secondary to metastasis of tumour to bone.

These experiments suggest that human joint fluid cells, unlike comparable numbers of rabbit peritoneal exudate cells (Kaiser and Wood, 1962), do not release pyrogen *in vitro* when incubated in saline. In addition, release of pyrogen does not normally occur when cells from either source are suspended in a buffered joint fluid or plasma medium.

Pyrogen is released from human blood cells following phagocytosis of heat-killed staphylococci (Bodel and Atkins, 1966). Since *in vitro* studies of the phagocytic capacity of joint fluid cells for staphylococci have shown little if any impairment of phagocytic function (Bodel and Hollingsworth, 1966), experiments were set up to determine whether pyrogen would be released from such leucocytes after phagocytosis of bacteria. As in the experiment shown in the upper curve of Fig. 1, release of pyrogen was often demonstrable following a phagocytic stimulus. In this part of the experiment, joint fluid cells were incubated in the saline medium with the addition of 20 per cent cell-free joint fluid and heat-killed *Staphylococcus aureus*, in a ratio of 12 bacteria to 1 leucocyte. In similar experiments, however, pyrogen could be recovered in significant amounts from cells of only 4 of 8 patients tested. Numbers of cells incubated were 4 7–$\times$ 10^7. This number of leucocytes obtained from blood and incubated under the same conditions regularly releases large amounts of pyrogen (Bodel and Atkins, 1967).

These results suggested that cells from joint fluid release less pyrogen than comparable numbers of blood granulocytes after the same stimulus. Blood and joint fluid cells were therefore obtained from 6 patients, and similar numbers of PMN from both sources were incubated with the same numbers of heat-killed staphylococci. Differentials on blood leucocytes were not performed in all patients, but previous studies as well as occasional counts in these studies have shown that 60–70 per cent of blood leucocytes prepared by our methods are

granulocytes. For these experiments the figure of 60 per cent was arbitrarily chosen for estimating numbers of blood granulocytes. Incubations of cells for 18 hr. were carried out in heparinized buffer with added glucose and 15–20 per cent cell-free plasma. Staphylococci were added to the same numbers of PMN cells from both synovial fluid and plasma, in a ratio of 5 bacteria to 1 PMN. After incubation, the supernatants from both joint fluid and blood cells were injected into the same 2 rabbits (into 1 rabbit in the experiment with patient A.G.), so that results from the 2 cell sources could be accurately compared. The maximum fevers in the 2 rabbits have been averaged in the Table. In each experiment it is clear that blood cells released more pyrogen than did corresponding numbers of joint fluid cells incubated under identical conditions. These data suggest, then, that joint fluid cells have a limited capacity to produce pyrogen even following a phagocytic stimulus.

TABLE.—*Mean Maximum Temperature in Pairs of Rabbits Injected with Material from Patients' WBCs*

Patient	Diagnosis	Supernatant from Joint fluid WBC and Staph △T*	Supernatant from blood WBC and Staph △T*
L. H.	Rheumatoid arthritis	0·05° (4·8)†	0·72° (4·0)†
A.K.	Rheumatoid arthritis	0·05° (3·8)	0·90° (3·1)
A. L.	Rheumatoid arthritis	0·18° (3·3)	0·98° (3·2)
V. Z.	Rheumatoid arthritis	0·70° (3·8)	1·10° (3·6)
J. G.	Rheumatoid arthritis	0·95° (4·5)	1·80° (3·8)
A. G.	Rheumatic fever	0·10° (3·9)	0·70° (3·8)

* Change in temperature above baseline
† PMN $\times 10^7$

To investigate this capacity in more detail, blood and joint fluid cells from 2 patients were obtained, incubated with bacteria as in the previous experiments, and aliquots of both cell suspensions removed after 6 hr. as well as after 18 hr. The results of 1 such experiment are shown in Fig. 2. Joint fluid and blood cells from a patient with rheumatoid arthritis were each incubated in buffer with 20 per cent plasma, and heat-killed staphylococci added in a ratio of 5 bacteria to 1 PMN. A portion of each mixture was removed at 6 hr.; the remainder was incubated for 18 hr. Since less pyrogen is usually released by blood cells at 6 hr. than at 18 hr., supernatant from larger numbers of cells was injected in the 6 hr. sample. Whereas nearly the same amount of pyrogen was released from this patient's joint fluid cells as from similar numbers of blood cells after 6 hr. of incubation, markedly less pyrogen was produced by the exudate cells than by the blood cells at 18 hr. The same results were obtained in the second experiment with cells from another patient. It appears, then, that the human synovial exudate cell has only a limited capacity to release pyrogen after a phagocytic stimulus, and that little or no additional release occurs after the first few hours of incubation.

Since it was conceivable that cells obtained from joint fluid might already have released pyrogen *in vivo*, it was also of interest to determine whether any pyrogen could be found in the cell-free joint fluid removed from these patients. Joint fluid was collected with heparin, and the cells removed by centrifuging at

3000 r.p.m. for 30 min. Supernatants were injected in volumes from 7–13 ml. per rabbit, frequently after being kept at 4° for several days, or at —20° for several weeks. Samples were re-centrifuged just before injection. For comparison, equal volumes of normal plasma and plasma from patients with arthritis were also

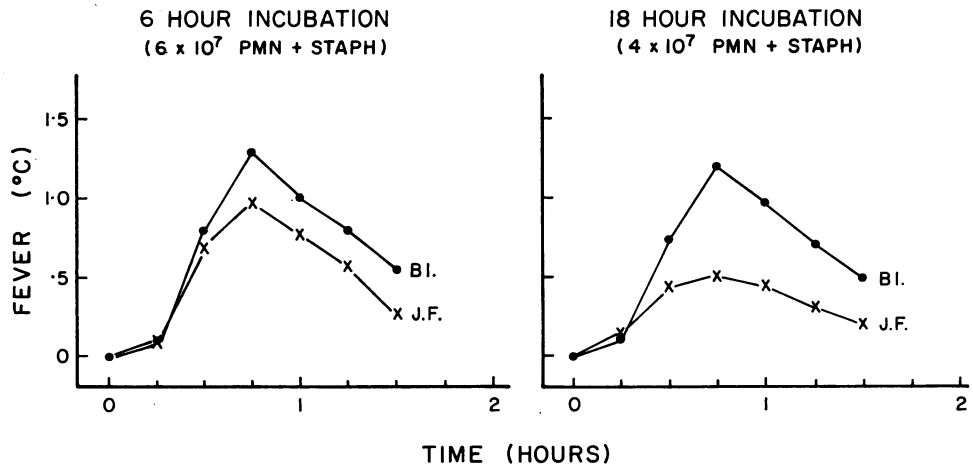

FIG. 2.—Fevers in the same rabbit to injection of supernatants from 6 or 18 hr. incubations of cells obtained from joint fluid (J.F.) and blood (Bl.) of a patient with rheumatoid arthritis. Numbers of polymorphonuclear leucocytes (PMN) from which the supernatant was derived for each injection are shown in parentheses.

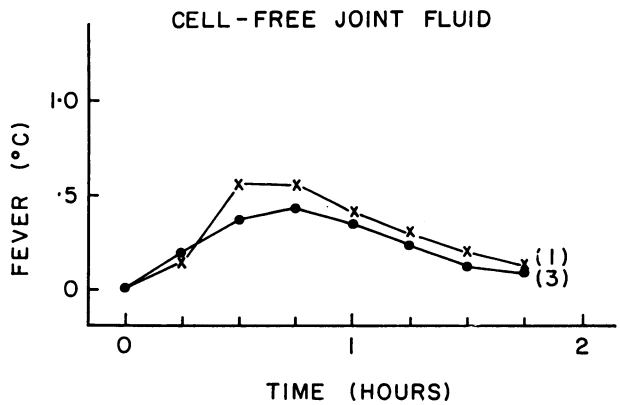

FIG. 3.—Average fevers in rabbits to injection of 10 ml. of cell-free joint fluid obtained from a patient with rheumatoid arthritis (x–x) and a patient with joint inflammation secondary to metastatic tumour (●–●). Numbers of rabbits are shown in parentheses.

injected. Positive results were considered to be a response of greater than 0·3° occurring 30–60 min. after injection. Such a rapidly acting pyrogen was detected in 13 joint fluid samples, obtained from 11 different patients. Two examples are shown in Fig. 3. Negative results were obtained with fluid from 5 patients. There seemed to be no relationship between the presence or absence of pyrogen and the type of inflammatory disease. A pyrogen was detected in the fluids of

6 patients with rheumatoid arthritis, 2 with rheumatic fever and 1 each with pseudogout, atypical Reiter's syndrome and metastatic tumour. On the other hand fluids from 3 patients with rheumatoid arthritis and 2 patients with osteoarthritis were non-pyrogenic. For comparison, plasma from 4 patients with rheumatoid arthritis, 1 patient with osteoarthritis, and 2 normal subjects was tested and did not contain pyrogen. In Fig. 4, a comparison between temperature responses produced by injection of joint fluid and plasma from the same patient is

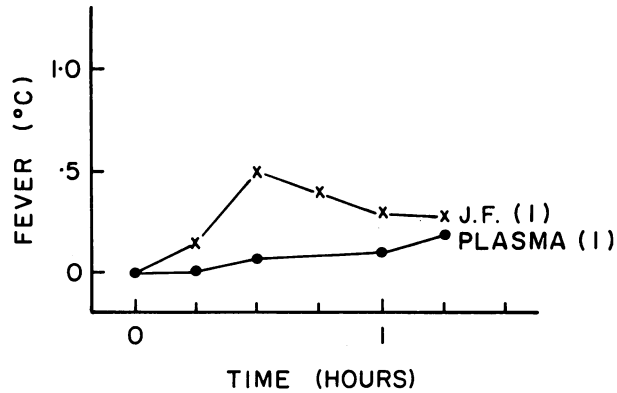

FIG. 4.—Temperature responses to injection of 10 ml. of cell-free joint fluid and plasma from a patient with atypical Reiter's syndrome. One rabbit received each injection.

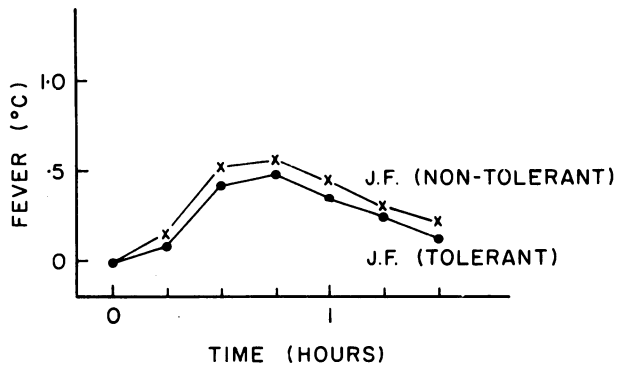

FIG. 5.—Average fevers in two rabbits to 15 ml. of cell-free joint fluid before and after induction of non-reactivity to endotoxin.

shown. Frequently, late pyrogenic responses occurred, beginning 60–90 min. after injection of plasma. This was occasionally observed with joint fluid also. Since such responses are known to occur following injection of a number of different agents into rabbits, but not after injection of endogenous pyrogen (Atkins and Snell, 1965), these late fevers were not considered relevant. They did not appear to represent hypersensitivity responses, and are as yet unexplained.

Although the shape of the early fever curves produced by most joint fluid samples suggested that endogenous pyrogen was present, more evidence was needed to exclude the possibility that small amounts of endotoxin could be responsible.

Sixty ml. of pyrogenic joint fluid were obtained by combining a group of individual fluids, and the mixture was stored at −20°. Two rabbits were tested with 13–15 ml. of this fluid. Each was then injected with 0·5 ml. of undiluted typhoid vaccine (Atkins and Wood, 1955). This dose of vaccine is known to prevent a febrile response to a small dose of endotoxin (producing 0·5–1·0° maximum fever) (Snell and Atkins, 1968a). The day after vaccine was given, both rabbits were shown to be fully reactive to the joint fluid (see Fig. 5), although they were unreactive to a small dose of endotoxin (1 ml. of 1 : 100 dilution of vaccine). These results indicate that the pyrogen in joint fluid is not endotoxin.

DISCUSSION

For many years it was thought that the pyrogen released from leucocytes, so-called " endogenous " pyrogen, was entirely species-specific. Since endogenous pyrogen from human blood cells has been found to be biologically active in rabbits (Bodel and Atkins, 1966), producing characteristic fever within 30–60 min. after i.v. injection, the release of pyrogen by human cells can now be assayed conveniently. The predominantly granulocytic exudate present in effusions from inflamed joints provides a readily available source of exudate in humans. Synovial exudate fluid and cells were studied for their pyrogen content and capacity for pyrogen production with techniques similar to those used to study peritoneal exudates of rabbits.

The human exudative cells, in contrast to similar cells obtained from rabbits (Kaiser and Wood, 1962), did not spontaneously produce measurable amounts of endogenous pyrogen when incubated in saline. They also did not release pyrogen, with the exception of cells from one patient, when incubated in a buffer medium containing glucose, joint fluid, or human plasma. This may represent a species difference in the behavior of leucocytes. However, rabbit peritoneal exudate contains a macromolecular substance (Wood, personal communication), perhaps bacterial endotoxin (Fruhman, 1964), that is known to activate cells to produce endogenous pyrogen, and may be responsible for the " spontaneously " active cells found in rabbit exudates (Hahn, Char, Postel and Wood, 1968). If this mechanism explains the difference between our findings with human cells and those previously described for rabbit exudate cells, such an activator may not be present in the joint fluids of patients with rheumatoid arthritis, pseudogout, and other inflammatory joint disease. On the other hand, if the human joint fluid cells in our studies are many hours older than those usually obtained from rabbit exudates, pyrogen production might reasonably be diminished. Recently, for example, release of pyrogen by rabbit exudate PMN incubated in saline has been shown to occur only for a few hr. (Hahn *et al.*, 1968). The tissue life span of human joint fluid PMN is not known, although the half-life in the joint fluid itself is calculated to be about 4 hr. (Bertino, Hollingsworth and Cashmore, 1963).

Blood leucocytes of both rabbits (Berlin and Wood, 1964*b*) and humans (Bodel and Atkins, 1966) produce pyrogen following phagocytosis of bacteria. Joint fluid leucocytes, which have been shown to engulf staphylococci *in vitro* as efficiently as do blood cells (Bodel and Hollingsworth, 1966), produced some pyrogen after phagocytosis of heat-killed staphylococci. However, they did not produce as much pyrogen as did comparable numbers of human blood leucocytes, due to a markedly impaired capacity to sustain pyrogen production over an 18 hr. period.

Diminished pyrogen production by joint fluid cells suggested the possibility that they had been activated previously and depleted of pyrogen, perhaps by some event occuring during the synovial inflammatory process. To investigate this possibility, cell-free joint fluid was tested for its pyrogenicity in rabbits. Significant early (30–60 min.) fever, characteristic of endogenous pyrogen was caused by 7–13 ml. of joint fluid in 13 experiments with fluid from 11 patients; 5 fluids did not contain measurable amounts of early acting pyrogen. Normal plasma and plasma patients with rheumatoid arthritis, injected in similar quantities, did not produce early fever. The shape of the fever curves in rabbits is identical to that of endogenous pyrogen produced by human blood leucocytes. Also, pyrogen in human joint fluid was active in endotoxin-refractory rabbits. Although chemical methods are needed to prove the identity of this pyrogenic material, these results suggest that the pyrogenic material in cell-free joint fluid is an endogenous pyrogen.

It seems possible that synovial exudate cells in inflammatory arthritis release endogenous pyrogen during the inflammatory response *in vivo* and are, therefore, deficient in pyrogen production either when incubated alone or when activated by phagocytosis *in vitro*. This explanation is consistent with the current hypothesis (Snell and Atkins, 1968b) that endogenous pyrogen is a small protein which is stored in granulocytes in an inactive form and released over a number of hr. following a variety of stimuli. The duration of any previous period in which the cell is " active " will, therefore, influence the subsequent capacity for pyrogen release. It is perhaps relevant that degranulation is often present in synovial exudate cells (Bodel and Hollingsworth, 1966), since loss of granules is a frequent accompaniment of cell activation by a number of different stimuli (Hirsch, Bernheimer and Weissmann, 1963).

SUMMARY

Leucocytes obtained from joint fluids of 11 patients with inflammatory arthritis did not release detectable amounts of pyrogen after incubation in saline. Further, pyrogen was obtained from cells of only one patient when incubation was carried out in a buffered joint fluid or plasma medium. Following phagocytosis, release of pyrogen could be detected from cells of 4 out of 8 patients. However, granulocytes from joint fluids uniformly produced less pyrogen than did granulocytes from blood.

Cell-free joint fluid from 11 patients contained a rapidly acting pyrogen which appeared to be an endogenous pyrogen.

These results suggest that granulocytes obtained from human joint fluids, unlike those previously studied from peritoneal exudates of rabbits, do not spontaneously release pyrogen during *in vitro* incubation. They further suggest that such cells may have been previously activated *in vivo*, releasing pyrogen into the joint fluid, and thereby reducing their subsequent capacity for production of pyrogen.

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REFERENCES

ATKINS, E. AND HEIJN, C. JR.—(1965) *J. exp. Med.*, **122**, 207.
ATKINS, E. AND SNELL, E. S.—(1965) 'The Inflammatory Process.' New York (Academic Press), p. 495.
ATKINS, E. AND WOOD, W. B. JR.—(1955) *J. exp. Med.*, **101**, 519.
BENNETT, I. L. JR. AND BEESON, P. B.—(1953) *J. exp. Med.*, **98**, 493.
BERLIN, R. D. AND WOOD, W. B. JR.—(1962) *Trans. Ass. am. Physns*, **65**, 190.—(1964*a*) *J. exp. Med.*, **119**, 697.—(1964*b*) *J. exp. Med.*, **119**, 715.
BERTINO, J. R., HOLLINGSWORTH, J. W. AND CASHMORE, A. R.—(1963) *Trans. Ass. am. Physns.*, **76**, 63.
BODEL, Phyllis. AND ATKINS, E.—(1966) *Proc. Soc. exp. Biol. Med.*, **121**, 943.—(1967) *New Eng. J. Med.*, **276**, 1002.
BODEL, Phyllis. T. AND HOLLINGSWORTH, J. W.—(1966) *J. clin. Invest.*, **45**, 580.
FRUHMAN, G. J.—(1964) *Ann. N.Y. Acad. Sci.*, **113**, 968.
HAHN, H. H., CHAR, D. C., POSTEL, W. B. AND WOOD, W. B. JR.—(1968) *J. exp. Med.* In press.
HIRSCH, J. G., BERNHEIMER, A. W., AND WEISSMANN, G.—(1963) *J. exp. Med.*, **118**, 223.
KAISER, H. K. AND WOOD, W. B. JR.—(1962) *J. exp. Med.*, **115**, 27.
SNELL, E. S. AND ATKINS, E.—(1968a) *Am. J. Physiol.*, **212**, 1103.—(1968b) In '*The Biologic Basis of Medicine.*' Ed. R. A. Farrand. London (Academic Press). In press.