In 30 animals there was only one where the tumor growth could be demonstrated radiologically as expanding and destroying the bone in the area adjacent to the knee joint, *i.e.*, the lower femur. The breast tumor tended to break out locally from the confines of the bone and did not show the sponge-like expansion seen with fibrosarcoma.

Of all animals undergoing irradiation for the established intrafemoral breast tumor, two features are of interest. First, despite 3000 r to the tumor cells in the leg of a rat, the transplanted tumor was not killed but an immense fibrous reaction occurred about it. The extensive involvement of the lungs in the irradiated animals (Fig. 4) was much in excess of those undergoing oophorectomy although 2 of the latter had small nodular metastases. One would have postulated that if the cells had reached the lungs at the time of the original inoculation then both groups would contain metastases of equal size. It is proposed to pursue this problem further by removal of the left limb immediately after the surgery, and at intervals thereafter in an attempt to show at what phase in the experiment the lungs became involved. It may be that conditioning in an osseous environment enhances metastases to the lungs, or irradiation to the femur may retard the advance of tumor at this site and allow time for the cells that have metastasized to the lungs to appear.

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Reversal of Chemotaxis *in vitro* and Chemotactic Activity of Leukocyte Fractions.* (31263)

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Boyden(1) described an *in vitro* technique for measuring quantitatively leukocyte chemotaxis. This method involved inducing granulocyte migration through a filter membrane and counting the cells which reached the lower surface of the filter after a set time interval. Boyden's observation that an active substance placed on both sides of the membrane prevented significant migration suggested that a concentration gradient of chemotactically active substance across the filter membrane was required. The first part of the present study was undertaken to investigate further if chemotaxis, as measured by Boyden's technique, is dependent upon leukocyte response to a continuous concentration gradient of an active substance. Thus, the effect of reversing the concentration gradient of chemotactic serum on leukocytes in the process of migration was determined.

Boyden(1) demonstrated that fresh serum contained a heat labile factor or factors which, when incubated with antigen-antibody complexes, would produce a heat-stable chemotactic agent. Recent studies using this technique have further characterized the conditions under which serum chemotactic substance is produced(2) and have investigated its relationship to serum hemolytic complement(3). These studies suggest that most substances have been demonstrated to have chemotactic activity *in vivo* exert it through a common humoral mechanism. The presence of chemotactic materials in cells or tissues with activity independent of serum is un-

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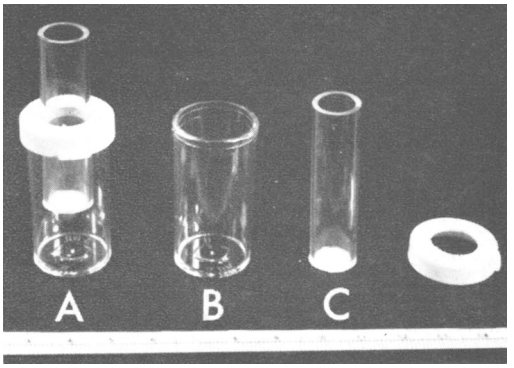

FIG. 1. Assay of chemotaxis. A = assembled chamber. B = test solution compartment. C = cell suspension compartment.

certain. The investigations of Hurley(4), however, indicate that there is chemotactic activity associated with leukocyte extracts. Janoff and Zweifach(5) have demonstrated that a polycationic protein in fresh serum from rabbit leukocyte lysosomes produces, at least *in vivo*, an inflammatory response with accumulation of polymorphonuclear leukocytes. The second part of this investigation was designed to determine if isolated fractions of polymorphonuclear leukocytes possess chemotactic activity *in vitro* in the absence of fresh serum.

Materials and methods. Adult New Zealand white rabbits of both sexes served as the source of serum and cells. Fresh normal rabbit serum was used as the source of chemotactic factor after incubation for 1 hour at 37°C with 1 mg/ml zymosan. The zymosan was removed by centrifugation and the supernatant serum was then heated at 56°C for 30 minutes. Serum treated in this manner is designated chemotactic serum (Cx-NRS). Test solutions contained 20% Cx-NRS in Hanks' balanced salt solution (BSS).

Fresh, 20% normal rabbit serum, heated at 56°C for 30 minutes (HT-NRS) was used in Hanks' BSS as nonchemotactic medium. All BSS contained 50 units/ml penicillin, 0.25 mg/ml streptomycin, and 0.01 mg/ml heparin.

Polymorphonuclear leukocytes were obtained by the method of Hirsch and Church (6). The cell suspension was adjusted to 1.2-1.8 $\times$ 10^6 cells/ml in 20% HT-NRS.

Cell fractions of 0.8 $\times$ 1.5 $\times$ 10^9 leukocytes were obtained by the method of Cohn and Hirsch(7), and the granule fraction was lysed by alternately freezing and thawing 10 $\times$. The granule membranes were removed by centrifugation at 18,000 g(7). The volume of 0.34 M sucrose varied from 6-10 ml, depending upon the amount of test solution needed for each experiment. All fractions tested for chemotactic activity were dialyzed in the cold against 200 ml Hanks' for at least 6 hours to remove most of the sucrose. The solutions were kept at 0-2°C until tested within one to 3 days.

The assay of chemotaxis was essentially that of Boyden(1). The cells were incubated aerobically at 37°C for 3 hours. A modified chamber was used (Fig. 1). The bottom compartment, *i.e.*, the test solution compartment, was a 3-dram plastic pill vial. The top compartment, which contained the cell suspension, was a 4 cm long lucite tube of 1 cm diameter which fitted snugly into a hole drilled in the plastic cap of the vial. A Millipore® filter of 3 μ mean pore diameter and thickness of 160 μ was cemented to one end of the tube to form the cell suspension compartment. This arrangement allowed the cell suspension compartment to be removed during the incubation period with minimal disturbance of the cells.

The number of cells in either 5 or 10 randomly selected high power fields ($\times$ 450) from different areas of the filter was determined and the average per field was computed. Only those cells which had reached the lower surface of the filter membrane were counted. Duplicate chambers were employed in all experiments and control chambers, both positive (20% Cx-NRS) and negative (20% HT-NRS), were included to indicate the relative responsiveness of the cell suspension. Positive control chambers usually resulted in the migration of more than 300 cells per field. It was not considered necessary to determine the exact number of cells when the migration was of this magnitude.

Results. Effect of reversal of concentration gradient of chemotactic serum on migrating polymorphonuclear leukocytes. Reversal of the concentration gradient was effected in the following manner. During incubation of leukocytes in a chamber containing Cx-NRS, it is assumed that chemotactic factor diffuses

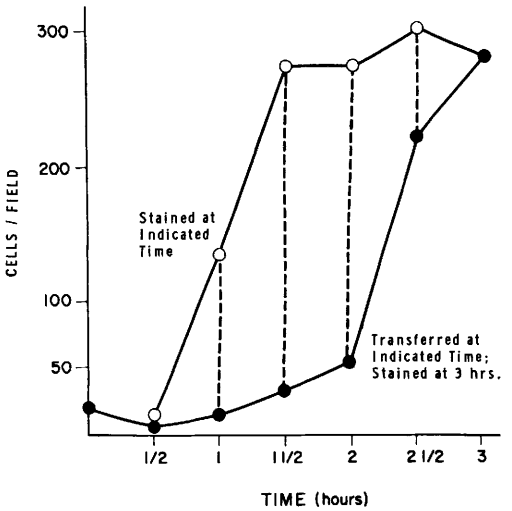

FIG. 2. Reversal of chemotaxis. ○ Cells/field on lower surface of filter at times of transfer. Test solutions: .4 ml Cx-NRS + 1.6 ml Hanks' solution. ● Cells/field remaining on lower surface of filter after transfer of cell compartment and incubation for remainder of 3-hr period, as described in text. Test solutions: before transfer, .4 ml Cx-NRS = 1.6 ml Hanks' solution; after transfer, .4 ml HT-NRS + 1.6 ml Hanks' solution.

into the cell suspension compartment, but that a gradient is maintained sufficient to promote the observed migration. After incubation for a time which should allow diffusion of the factor into the cell compartment, this compartment is transferred to another test compartment containing HT-NRS. The cells are thus exposed to a reversed concentration gradient of chemotactic serum.

The results of one representative experiment are given in Fig. 2. They demonstrate that the cells, after responding to an initial concentration gradient of Cx-NRS by migrating 160 μ to the lower side of the filter, are capable of reversing their direction to migrate toward the upper surface.

The number of cells which actually reversed direction of migration following transfer to non-chemotactic serum may be seen in Fig. 2 by comparing the number of cells found on the filter stained at the time of transfer to those found on the filter stained after incubation in the HT-NRS test solution. The total incubation time for all transferred compartments was 3 hours. The largest difference in cells per field between control chambers and transferred chambers occurred when cells were transferred after 1½ hours. This allowed a sufficient time for diffusion to occur, as well as effective time for reversed migration.

A control experiment demonstrated no significant difference between positive control migration and the migration observed when the cell compartments were transferred to test compartments containing Cx-NRS. Thus, the manipulations necessary for the transfer do not merely dislodge the cells. Another experiment showed that the results are not due to random movement after the transfer from Cx-NRS to HT-NRS. Removing the cell compartment fluid and replacing it with 20% HT-NRS at the time of transfer to HT-NRS (1½ hours) did not result in a decrease in cells per field after 1½ hours of further incubation.

Table I shows the effect of a leukocyte homogenate in the test compartment to which the cell compartment was transferred after 1½ hours incubation in a chamber containing Cx-NRS. The homogenate was prepared from leukocytes obtained from the same peritoneal exudate which provided the cell suspension, but was concentrated to 5.4×10^6 cells/ml, homogenized by 10 strokes with a teflon pestle, and rapidly frozen and thawed 4 ×. The data indicate that cells in contact with such a homogenate are inhibited in reversing migration. Such inhibition would be expected to occur if the leukocytes contained chemotactic substance or substances which interfered with the gradient, as showed by the experiments reported below.

Chemotactic activity of fractions of 0.34 M sucrose-lysed polymorphonuclear leukocytes. The chemotactic activity of leukocyte fractions was determined. The results of testing 4 different preparations are shown in Table II. Experiments 1-3: The variability among these experiments with respect to a given fraction is largely due to the difference in the number of washes (given in parentheses) in Hanks' BSS. In the first experiment, the 250 × *g* supernate, consisting of BSS used to wash the cells from the peritoneal cavity, was tested. This solution showed no chemotactic activity and was not included in further studies. The 400 × *g* pellet (nuclear pellet) exhibited some activity, which decreased with washing and was attributed to contamination

by the lysosomal fraction. The post-granule supernate, containing free soluble cell constituents, showed no activity. The granule (lysosome) lysate supernate consistently stimulated migration, but to considerably lesser extent than chemotactic serum. The granule membrane pellet was chemotactic; this activity decreased with washing. Experi-

TABLE I. Inhibition of Reversal of Migration by a Leukocyte Homogenate.

Test compartment before transfer	Incubation time (hr)	Test compartment after transfer	Incubation time (hr)	Cells/field (duplicate chambers)
A. Cx-NRS	3	—	—	300 264.0
B. Cx-NRS	1½	—	—	255.4 300
C. Cx-NRS	1½	HT-NRS	1½	35.0 28.6
D. Cx-NRS	1½	Leukocyte homogenate	1½	183.8 117.0
E. HT-NRS	3	—	—	10.6 30.2

Test solutions:
A. .4 ml Cx-NRS + 1.6 ml BSS
B. .4 ml Cx-NRS + 1.6 ml BSS
C. Before transfer: .4 ml Cx-NRS + 1.6 ml BSS
After transfer: .4 ml HT-NRS + 1.6 ml BSS
D. Before transfer: .4 ml Cx-NRS + 1.6 ml BSS
After transfer: .4 ml HT-NRS + 1.6 ml leukocyte homogenate
E. .4 ml HT-NRS + 1.6 ml BSS

TABLE II. Chemotactic Activity of Fractions of 0.34 M Sucrose-Lysed Polymorphonuclear Leukocytes.

Test compartment	Experiment number 1	2	3	4
	Cells/field (duplicate chambers)			
Cx-NRS	>300 >300	>200 >200	77.7 90.3	>300 >300
250 × *g* supernate (peritoneal exudate)	3.0 0.6			
400 × *g* pellet (nuclear pellet)	14.4 (2)* 12.0	28.4 (2) 26.4	4.1 (5) 3.3	
8200 × *g* supernate (post-granule supernate)	9.2 8.0	7.2 8.4	10.8 6.6	
18,000 × *g* supernate (granule lysate)	39.4 28.8	45.2 50.4	36.0 44.6	A. 17.3 18.3
18,000 × *g* pellet (granule membranes)	10.4 (2) 6.2	60.0 (0) 125.2	26.6 (3) 12.0	B. 28.7 34.1
HT-NRS	1.2 3.8	5.6 4.2	2.7 2.5	8.8 6.6

Exp 1, 2, 3:
Test solutions: .6 ml cell fraction + .4 ml HT-NRS + 1.0 ml BSS
Cell suspensions: 1.0 ml of 1.2-1.8 × 10^6 cells/ml BSS, 20% HT-NRS

Exp 4:
A. Test solution: .2 ml HT-NRS + .8 ml cell fraction
Cell suspension: 1.0 ml of 2.6 × 10^6 cells/ml BSS, 20% HT-NRS
B. Test solution: 1.0 ml cell fraction
Cell suspension: 1.0 ml of 2.6 × 10^6 cells/ml BSS, 20% HT-NRS

* Number in parentheses refers to No. of washes in BSS.

ment 4: Since in the above experiments the test solution contained 20% HT-NRS, it was of interest to determine whether or not HT-NRS was essential for the chemotactic activity of the lysosome lysate. In this experiment (Table II), HT-NRS was omitted from the test solution, but no significant differences between the 2 test solutions were observed.

Discussion. This study has shown that leukocytes are able to reverse their direction of migration in response to an oppositely applied concentration gradient of chemotactic serum. This observation indicates that the active serum factor does not act irreversibly, and that the cells are continuously responsive to a gradient of active substance in this *in vitro* test.

The second part of the study has indicated that there is some chemotactic factor associated with the lysosomal fraction of rabbit leukocytes which does not require fresh serum for its activity. Ward *et al*(3) have reported in an addendum that neither lysosomal lysate nor the anti-inflammatory polycationic protein showed chemotactic activity *in vitro*. However, it is felt that differences in the assay of chemotaxis, particularly in the pore size of the filter employed, could be responsible for these conflicting results. Under the conditions employed in this laboratory, the lysosome-related factor induces less migration than the serum factor. It is not clear whether this is a result of concentration differences, or whether a more complex mechanism acts *in vivo* to produce the marked inflammation observed by Janoff and Zweifach(5).

Even though chemotactic activity has been associated with lysosomes, the possibility must be considered that the leukocytes ingested the active serum factor during glycogen stimulation *in vivo* and that it was localized in the lysosomal portion after fractionation of the harvested cells. All preparations of granules were obtained from cells which had been stimulated with glycogen.

Summary. Using Boyden's *in vitro* method, it was demonstrated that rabbit polymorphonuclear leukocytes reversed their direction of migration in response to a concentration gradient of chemotactic serum. The presence of a chemotactic factor or factors associated with the lysosomal fraction of leukocytes which does not require fresh serum for its activity was shown.

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Calcium Sensitivity of Cell Cultures Derived from Adenovirus-Induced Tumors.* (31264)

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Certain strains of human adenoviruses have been shown to induce tumors in hamsters(1), rats(2) and mice(3). Cells from these tumors were transplantable(2) and contained virus-specific complement-fixing and fluorescent

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