ANALYSIS OF CHEMOTAXIS IN WHITE BLOOD CELLS

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ABSTRACT We wish to report the development of an assay system for the study of white blood cells in vitro. With this system we have demonstrated that a yet unidentified substance found in red blood cell membranes and cyclic adenosine monophosphate (cAMP) cause the chemotactic response in white blood cells. We have not yet determined whether the substance released from the membrane is cAMP.

INTRODUCTION

Chemoreceptors are widely distributed in nature ranging from the primitive virus to the multistructured olfactory bulb found in the higher vertebrates. Julius Adler and his coworkers (1), working with *Escherichia coli*, have found five specific chemoreceptors that when stimulated caused a specific response called the avoidance reaction (shock reaction).

One of the more well-known chemotactic "organisms," the white blood cell, has yielded to date far less quantitative data than *E. coli* and such questions as the number of receptors or even the specificity and identity of the chemotactic substance are still unanswered. It is clear from the work of Harris (2) and Gowland (3) that wound tissue releases a chemotactic substance or substances that attract white blood cells. There exists evidence that at least two substances may be involved, a heat-stable dialyzable molecule and a heat-labile nondialyzable substance (3). For the most part, most chemotactic experiments have been conducted by making a skin lesion and then observing the number of white blood cells arriving at the lesion as a function of test substances placed on the lesion. In the white cell in vitro system several agents have been shown to cause a positive chemotactic response but in general these substances have been rather ill-defined macromolecules (4, 5).

Using either a laser or UV microbeam, Bessis and Burte (6) have found that a laser-damaged red blood cell releases a substance that attracts white blood cells within a radius of 500 μ. The strongest responses occur with the neutrophils, response intensities decreasing to the eosinophils and the monocytes, with the lymphocytes

showing no chemotaxis. Based on time response studies, Hu and Barnes, by modeling a diffusion equation, have calculated that the chemotactic substance should have a molecular weight no greater than 10,000 (7). It is not clear from the published reports whether the white blood cells are demonstrating phobotaxis as in bacteria or topotaxis.[1] Bessis (6) himself describes the motion of the leukocytes as they approach the target "to vary from a straight line to a highly tortuous path."

We wish to report here that we have developed a technique that allows us to measure the specific direction of movement of a great number of white blood cells in vitro after an imposed chemical gradient. We have found that the direction of movement is towards isolated red blood cell membranes. In addition, we wish to report that cyclic adenosine 3′5′-monophosphate (cAMP) is a strong chemotactic agent.[2] We have not determined whether the substance released from the red blood cell membranes is indeed cAMP.

MATERIALS AND METHODS

In all experiments reported here, sodium-citrated horse blood supplied by Colorado Serum Co. (Denver, Colo.) was used.

Preparation of White Blood Cells

10 ml of fresh horse blood were drawn into a syringe and the syringe was allowed to stand upside down in a 4°C refrigerator for about 1 hr. The serum on top and the buffy coat of white blood cells was pushed out of the syringe into a serum bottle. This was then centrifuged for 10 min at 1000 rpm (126 *g*) in a refrigerated Sorvall (Ivan Sorvall, Inc., Norwalk, Conn.) after which half of the serum was poured off leaving 2 ml of white blood cells in serum suspension.

Preparation of Serum

The serum was obtained by centrifuging 20 ml of blood for 10 min at 1000 rpm (126 *g*) and then decanting off the serum.

Clotting Procedure for White Blood Cells

2 ml of concentrated white blood cells and serum was placed in a 3.5 cm plastic Petri dish and clotted by titration with 0.01 M calcium chloride. Circular pieces of clot of approximately the diameter of 0.5 cm were cut out and then blotted on filter paper to remove excess serum and excess white blood cells.

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[1] If an organism responds to a stimulus by moving directly towards or away from the stimulus, it is called topotactic. If an organism merely stops its motion when it is going into a direction counter to the gradient, it is called phobotactic.

[2] This is in total agreement with a recent report from Bonner's group (8) in which they report positive chemotaxis of white blood cells as a result of cAMP; it is important to note that their method of assay differed substantially from our method.

Preparation of cAMP

cAMP purchased from Calbiochem (Los Angeles, Calif.) was diluted in our standard salt medium and then added to 5-μl capillaries (microcap pipette) to a final concentration of 6.25 μg/5 μl (4×10^{-3} M). This microcap pipette is now ready to be placed into our chamber.

Preparation of Medium

In all experiments, minimum essential medium (Eagle) with Hanks's salts purchased from Grand Island Biological Co. (Grand Island, N.Y.) was used. This medium was then fortified with 10% horse serum and 1% pencillin plus streptomycin solution (Grand Island Biological Co.) in all cases.

Preparation of Experimental Chambers

All chambers were constructed from ordinary precleaned microscope slides that had been previously coated with horse serum. The slide was divided into two rectangular sections by means of paraffin (Fig. 1).

Preparation of Red Blood Cell Membranes

Horse red blood cells were lysed by osmotic shock and the membranes pelleted by centrifugation for 30 min at 10,000 rpm (12,000 *g*). This pellet was washed once with distilled water. The membranes were resuspended in horse serum which was used to fill a capillary; this capillary was then placed in the experimental chamber.

RESULTS

When a white blood cell clot is incubated for approximately 15 hr at 37°C, there appears a symmetrical white halo around the clot. This halo has been found to consist of leukocytes which have been identified both by phase-contrast microscopy and by specific leukocyte-staining techniques. Fig. 2 A is a low power microscope picture showing the control clot and the surrounding white blood cells. Fig. 2 B is the identical experiment except that a red blood cell membrane extract is placed in a

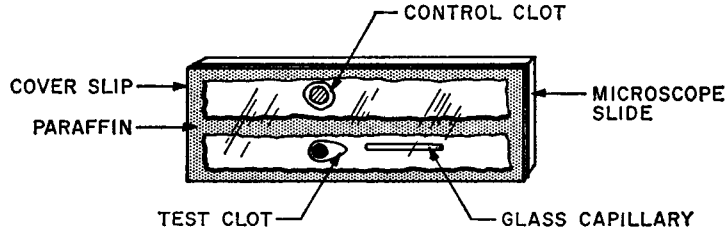

FIGURE 1 A sketch of the experimental chamber used to measure chemotaxis.

FIGURE 2 A photomicrograph of a serum clot and the white blood cells moving away. (A) Control clot with no chemotactic agent. (B) Positive chemotaxis towards a red blood cell membrane fraction. Arrow indicates direction of the gradient. (C) Positive chemotaxis towards a cAMP gradient, note the end of the capillary. × 11.

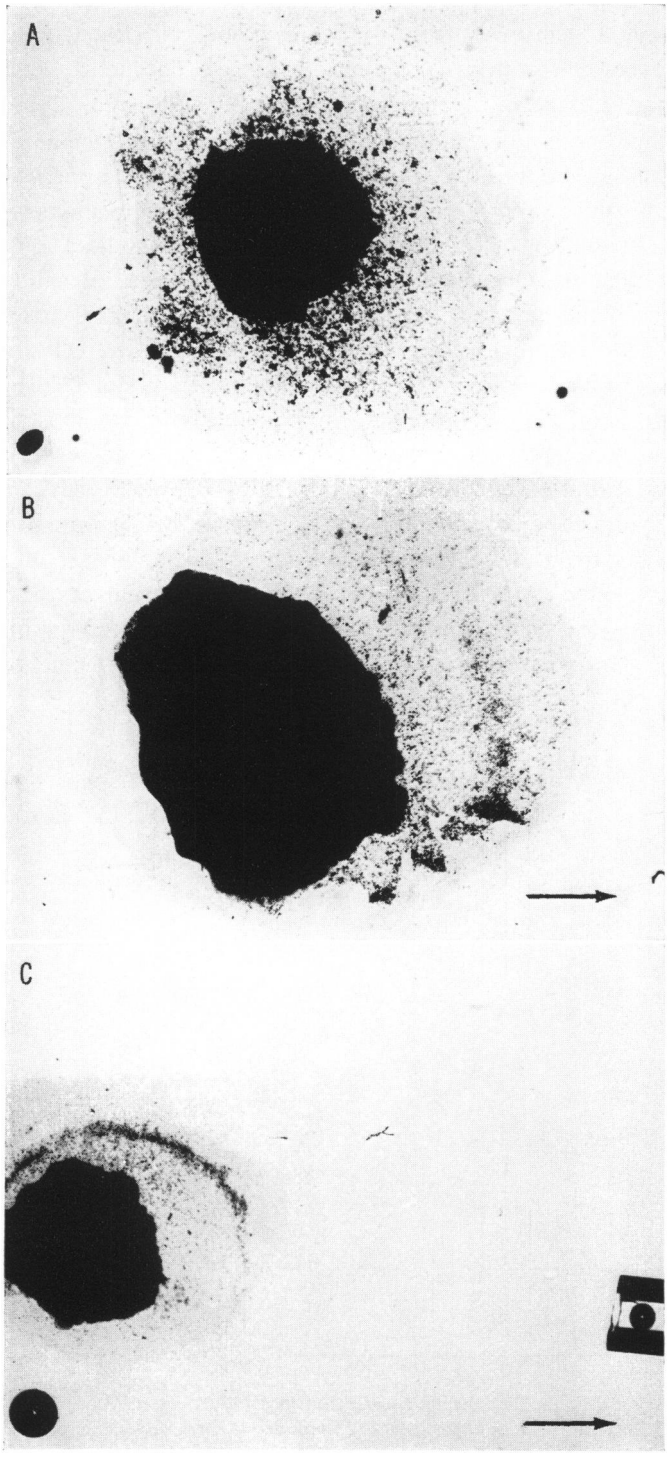

FIGURE 2

capillary about 0.5 cm away from the white blood cell clot. One can see that the white halo is skewed towards the capillary. In some experiments, there appears a line of white blood cells between the test substance and the white blood cell clot. The reason for this line is not yet known. Occasionally, a particular experiment with membranes shows no positive chemotaxis and therefore appears to be like the control, but we have rarely found a negative chemotactic response. Fig. 2 C shows the skewed distribution of cells towards the 5 μl capillary that contains 6.25 μg of cAMP (4×10^{-3} M). On a qualitative basis the response of white blood cells to cAMP has been the strongest and gives us the most reliable assay.

It has been noted previously that when measuring white cell chemotaxis there appears to exist a large variability from experiment to experiment (8). Because of this it is necessary to run a large number of experiments in order to substantiate a positive effect. Thus experiments were tabulated using the following criteria. 0 designates the uniform distribution of cells around the clot. + represents slight positive chemotaxis, ++, average chemotaxis, and +++, very strong chemotaxis. −1 represents movement away from the test substance. Fig. 3 is a plot of the number of experiments vs. the degree of the chemotactic response. It is clear that cAMP causes the largest response (Fig. 3 *b*). Although the response towards the red blood membrane fraction (Fig. 3 *c*) is less than towards cAMP, it is still significant. Membranes that

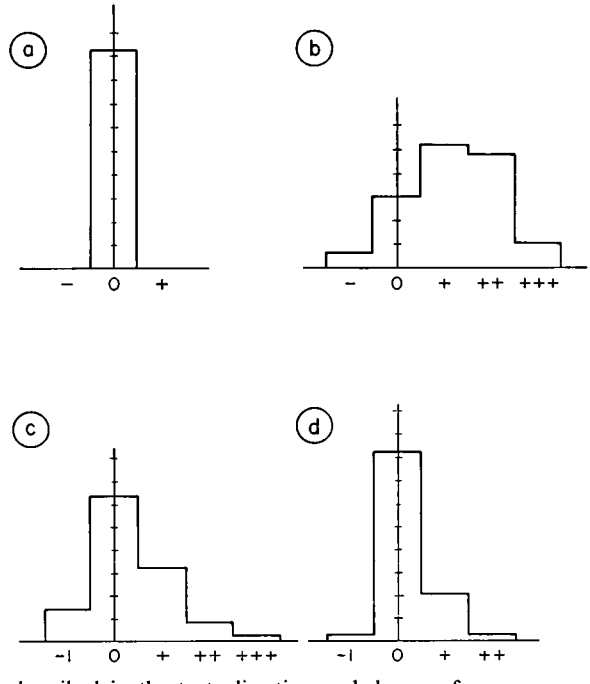

FIGURE 3 As described in the text, direction and degree of response were measured by eye. Fig. 3 is the number of experiments plotted as a function of the degree of the response. Each division of the ordinate axis represents five experiments.

have previously been dialyzed against phosphate buffer pH 7, for 5 hr appear to have a slightly reduced response (Fig. 3 *d*). The presence of a capillary by itself with no test agent gave the same results as chambers without capillaries, i.e., the standard control chambers.

DISCUSSION

One of the basic characteristics of a living system is its ability to respond to either internal or external environmental changes. To accomplish this living systems have developed an array of sensing devices which are specific to a battery of stimuli such as light, heat, mechanical displacement, and specific chemical substances. Receptors that respond to specific chemical substances are called chemoreceptors; chemotaxis is the term for the final response of systems, either moving into or away from the chemical gradient. The direction of the gradient is determined by one or more chemoreceptors. In higher organisms, with a well developed nervous system, the chemical receptors appear to work always as transducers. A molecule of a specific configuration attaches to the membrane surface and as a result of this specific attachment, a change in the membrane permeability occurs which then results in a change in the transmembrane potential. Any change of electrical potential occurring at a receptor as a result of a stimulus is called a receptor potential. The postsynaptic membrane of a chemical synapse is an example of a well developed chemoreceptor.

One of the most primitive chemical receptors known is found near the baseplate of the bacteriophage T4B. After six molecules of L-tryptophan combine with the T4B bacteriophage, the probability of finding the viral tail fibers extended is dramatically increased (19). A mechanism recently proposed is that the binding of the L-tryptophan functions to lower, by 50%, the activation energy required for a conformational change needed to extend the fibers (10). Thus the whole response can be explained in terms of a simple conformational change in one or more protein molecules.

In more advanced systems, such as the active movement of a eukaryotic cell towards a particular chemical agent, this response certainly must be a result of a series of complex biochemical steps. The fact that cAMP has been implicated in a myriad of biochemical reactions makes it difficult to pinpoint the specific kind of biochemical steps involved. To date cAMP has been shown to be involved directly or indirectly in the following cellular and intercellular events:

(*a*) cAMP has long been known to be an activator for phosphorylase kinase b (11).

(*b*) It has been shown that cAMP is necessary for transcription in both prokaryotes (12) and eukaryotic systems.

(*c*) Evidence, although less direct than the above, has been reported that cAMP is necessary for translation (13).

(*d*) cAMP has been shown to increase the cell permeability to small molecules such as water, urea, and sodium (14, 15). Perhaps the most interesting permeability effect is the immediate efflux of Ca^{++} after the addition of cAMP (16).

(*e*) cAMP is also needed for the formation of flagella in coli-form bacteria (17).

(*f*) Perhaps most relevant to our discussion is the fact that cAMP is the chemotactic substance released by the amebas of *Dictyostelium discoideum* that is directly responsible for the aggregation reaction (18).

(*g*) In addition, cAMP has been implicated in the formation of microtubules. Malaisse et al. (19) have shown the necessity of cAMP for the insulin release from the beta cells of the pancreas which is at least partly because of the interference of microtubule formation. Hsie and Puck (20) have found that cAMP when added to Chinese hamster cells causes them to elongate, and they have postulated that this is a direct result of excess microtubule formation. The fact that the elongation is inhibited after the addition of Colcemid strongly substantiated their arguments.

The chemotactic response to a cAMP gradient may certainly be a result of one or more of the above biochemical systems but it appears to us that the stimulation of microtubules may be the most important one. This follows from the fact that cell movement (ameboid movement) has been generally explained as a result of microtubule formation.

In addition, cAMP has been directly implicated in the phosphorylation reaction of the serine residues of neurotubular subunits isolated from bovine cerebral cortex (21).

The fact that both soil amebas and mammalian cells show chemotaxis towards a cAMP gradient led us to ask the question whether *E. coli* were also chemotactic to cAMP (a prokaryote in contrast to a eukaryote). Using a strain of bacteria, AW 405, that shows strong chemotaxis to some sugars and amino acids we found no chemotaxis as a result of a cAMP gradient.[3]

We feel the foregoing results open the door for a variety of important experiments on the nature of chemical communications in biological systems. In particular, our assay techniques should make quantitative measurements of leukocyte response to chemical stimuli possible in such a way as to facilitate studies in wound healing processes and leukemia. We also believe that further observations of the effect of cAMP on leukocytes will lead to a better understanding of the function of this compound in biological systems.

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BIBLIOGRAPHY

1. ADLER, J. 1969. *Science (Washington).* **166**:1588.
2. HARRIS, H. 1953. *J. Pathol. Bacteriol.* **66**:135.
3. GOWLAND, E. 1964. *J. Pathol. Bacteriol.* **87**:347.
4. KELLER, H. U., and E. SORKIN. 1968. *Experientia.* **24**:641.
5. BRIER, A. M., R. SNYDERMAN, S. E. MERGEHAGEN, and A. L. NOTKINS. 1970. *Science (Washington).* **170**:1104.

[3] Gamow, R. I., and B. Böttger. Unpublished results.

6. BESSIS, M., and B. BURTE. 1965. *Tex. Rep. Biol. Med.* **23**:204.
7. HU, C.-L., and F. S. BARNES. 1970. *Biophys. J.* **10**:958.
8. LEAHY, D. R., E. R. MCLEAN, JR., and J. T. BONNER. 1970. *Blood J. Hematol.* **36**:52.
9. STENT, G. S., and E. L. WOLLMAN. 1950. *Biochim. Biophys. Acta.* **6**:307.
10. GAMOW, R. I. 1969. *J. Virol.* **4**:113.
11. WALSH, D. A., J. P. PERKINS, and E. G. KREBS. 1968. *J. Biol. Chem.* **243**:3763.
12. SILVERSTONE, A. E., B. MAGASANIK, W. S. REZNIKOFF, J. H. MILLER, and J. R. BECKWITH. 1969. *Nature (London).* **221**:1012.
13. PASTAN, I., and R. L. PERLMAN. 1969. *J. Biol. Chem.* **244**:2226.
14. ORLOFF, J., and J. S. HANDLER. 1961. *Biochem. Biophys. Res. Commun.* **5**:63.
15. ORLOFF, J., and J. S. HANDLER. 1962. *J. Clin. Invest.* **41**:702.
16. FRIEDMANN, N., and C. R. PARK. 1968. *Proc. Nat. Acad. Sci. U.S.A.* **61**:504.
17. YOKOTA, T., and J. S. GOTS. 1970. *J. Bacteriol.* **103**:513.
18. KNIJN, T. M., D. S. BARKELEY, Y. Y. CHANG, and J. T. BONNER. 1968. *Amer. Midland Natur.* **102**:225.
19. MALAISSE, W. J., F. MALAISSE-LAGAE, and D. MAYHEW. 1967. *J. Clin. Invest.* **46**:1724.
20. HSIE, A., and T. T. PUCK. 1971. *Proc. Nat. Acad. Sci. U.S.A.* **68**:358.
21. GOODMAN, D. B., H. RASMUSSEN, F. DIBELLA, and C. EARL GUTHROW, JR. 1970. *Proc. Nat. Acad. Sci. U.S.A.* **67**:652.