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Cellular Immunity In Vitro: Migration Inhibition and Phagocytosis

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The activity of peritoneal exudate cells from candidin-sensitive and normal guinea pigs with and without antigen was studied in vitro with the aid of time-lapse, phase-contrast cinemicrography. Macrophages from normal animals migrate well on an agar medium and readily phagocytose Merthiolate-killed *Candida albicans* cells. A reduction in the migration of peritoneal macrophages from guinea pigs sensitized to *C. albicans* during the first 24 hr after exposure to antigen was accompanied by a decrease in phagocytosis of *C. albicans* cells. The macrophages were viable but comparatively immotile. Since the sensitized macrophages came from resistant donors, it is possible that the initial stage of cellular immunity to *C. albicans* is associated with a reduced activity of the phagocytic macrophages, apparently to limit spread of the pathogen from the infected area.

Cellular immunity and delayed hypersensitivity have been associated with acquired resistance to various infectious diseases (5). Herein, an increase in resistance to *Mycobacterium tuberculosis*, as indicated by a decrease in the numbers of viable organisms in the animal tissue, was accompanied by delayed hypersensitivity, as indicated by a simultaneous increase in diameter of the mouse footpad after injection of specific antigen. In the development of this state, mononuclear cells were prominent in the areas of infection and in the areas of footpad testing.

Delayed hypersensitivity is characterized in vivo by an infiltration of mononuclear cells into the skin test site of hypersensitive animals and in vitro by an altered activity of lymphocytes and macrophages; neither process is associated with conventional circulating antibody. Delayed hypersensitivity in vitro may be expressed as an inhibition of migration of macrophages from sensitized hosts in the presence of specific antigen (3, 8). This inhibition is caused by the release of soluble factor or factors from sensitized lymphocytes in the presence of antigen. In addition to inhibition of migration, the antigen-induced, soluble supernatant fluid from sensitized lymphocytes induces other responses in normal cells, such as aggregation (11), chemotaxis (15), and cytolysis (4). The intradermal injection of soluble supernatant fluid into normal guinea pigs produces an infiltration of mononuclear cells into the skin area (1).

The inhibition of macrophage migration was found to be associated with a decrease in phagocytic activity during the first 18 to 24 hr after exposure of normal macrophages to sensitized lymphocytes and antigen (9). This property of decreased phagocytosis of *Candida albicans* was unexpected, since the sensitized lymphocytes and macrophages were obtained from guinea pigs which had increased resistance to *C. albicans*.

A study was therefore initiated to investigate further the in vitro activity of lymphocytes and macrophages from hypersensitive guinea pigs with regard to both migration and phagocytosis. Peritoneal exudate cells from normal and sensitized guinea pigs were placed on an agar surface (10) with dead *C. albicans* cells, and their behavior in the presence or absence of soluble antigen was studied by time-lapse cinematography with a phase microscope. The observations revealed that the peritoneal exudate cells which were inhibited in their migration in vitro within 24 hr after addition of specific antigen phagocytosed *C. albicans* to a lesser extent.

MATERIALS AND METHODS

Guinea pigs of the Hartley strain, weighing 350 to 400 g, were sensitized by intraperitoneal inoculation of three to four doses each of 10^7 live *C. albicans* cells administered several weeks apart. Strain 3148 of *C. albicans* (9) was grown in Casamino broth (Difco) for 24 hr on a rotating shaker at 37 C. For use in phagocytosis, the cells were killed by exposure to 1:5,000 Merthiolate for 72 hr at 37 C and then washed three times with Hanks solution. The fungus cells were counted on a Levy hemacytometer before the appropriate number of cells was injected. For the induction of cells in the peritoneal cavity, 20 ml of

light mineral oil was injected intraperitoneally 5 to 7 days before the guinea pig was sacrificed. The resulting exudate contained approximately 60 to 70% macrophages and 30 to 40% lymphocytes. The cells were suspended in Hanks solution, washed twice, and packed by centrifugation at 800 rev/min for 10 min. Such cells did not yield *C. albicans* on culture on Sabouraud agar.

The studies on migration inhibition in which actual distances of migration were measured were carried out in 30-ml Falcon plastic tissue culture flasks. Time-lapse, phase cinephotomicrography of cell migration was carried out in sterile Sykes-Moore tissue culture chambers (Bellco Biological Glassware, Vineland, N.J.) fitted with no. 1 cover slips and silicone rubber gasket rings. A supporting medium contained 0.5% Ionagar no. 2, Hanks solution, 20% normal guinea pig serum, 100 units of penicillin/ml and 100 μg of streptomycin/ml, with or without soluble antigen, and was allowed to solidify on a cover slip. A tiny drop containing a suspension of 10^7 dead *C. albicans* cells was allowed to spread over the agar surface. The mononuclear cells, having been concentrated by centrifugation at 4 C for 10 min at 800 rev/min, were planted as a tiny drop on the nutrient agar. Chambers were closed with a second (bottom) cover slip and incubated at 37 C for 12 to 24 hr. To determine the viability of the cells, a drop of 1:2,000 neutral red was added onto the agar.

Time-lapse cinemicrography was performed with a Leitz Ortholux phase-contrast microscope attached to a Bolex 16-mm camera driven by a Sage cinemicrographic timer and motor. During periods of study, the culture chambers were maintained at 37 C with a Sage air curtain incubator. Kodak Plus-X reversal 16-mm black and white film was exposed at a speed of eight frames per min and at 100 ×, 250 ×, or 440 × magnification.

RESULTS

Normal phagocytosis in vitro. Phagocytosis was studied with cells from the peritoneal exudate of 21 normal Hartley guinea pigs. The behavior

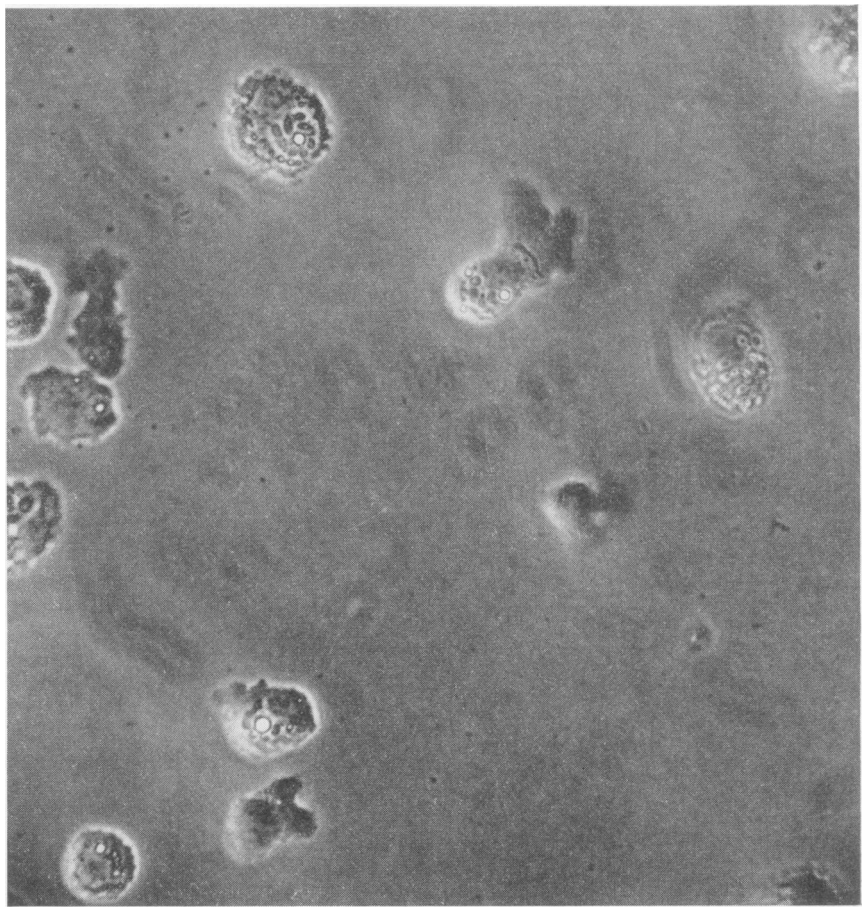

FIG. 1. *Phagocytosis of C. albicans by macrophages from sensitized guinea pigs in the absence of antigen. Note active macrophages containing many C. albicans and absence of C. albicans extracellularly on surrounding medium. Macrophages after extensive phagocytosis of C. albicans become less mobile.* × 700.

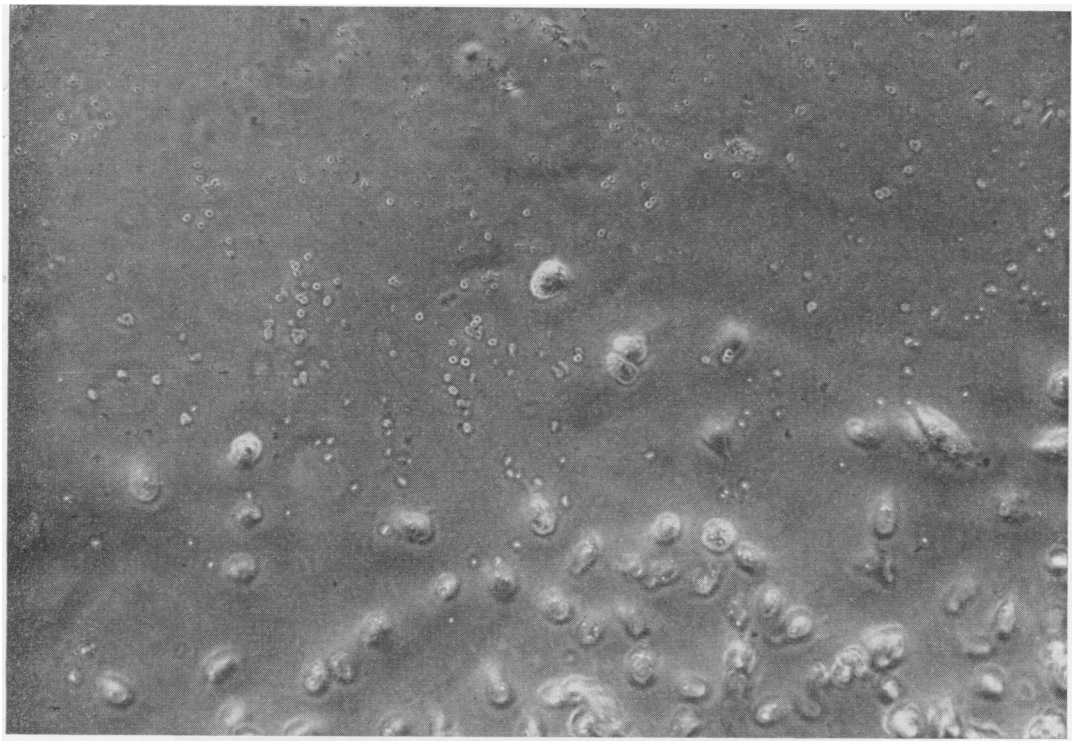

FIG. 2. *Phagocytosis of C. albicans by macrophages from sensitized guinea pigs in the absence of antigen. Note macrophages migrating upwards from bottom of photograph and phagocytosing C. albicans on the agar surface.* × *340.*

of such cells was observed directly on the agar medium which had been overlaid with Merthiolate-killed *C. albicans* cells. The migrating macrophages had a characteristic amoeboid Motion, in which large, broad pseudopodia and smaller, sharper filapodia were prominent. The peritoneal macrophages were constantly moving over the agar in a random fashion, did not adhere to each other, and did not interfere with each other's migration, although frequent contact was observed. The motion of the lymphocytes was readily distinguishable, in that they migrated more rapidly than the macrophages and had a prominent cytoplasmic process, the uropod, pointed away from the direction of motion.

As the normal macrophages migrated over the agar surface, they were very actively phagocytic on the *C. albicans* (Fig. 1). The pseudopodia, so important in the movement of the cells, were also very active in the engulfment of the yeast. Large vacuoles were immediately visible about the ingested fungus cell, but were less obvious as the *C. albicans* was slowly digested.

As the macrophages migrated out from the inoculation site, they "cleaned" the agar of *C. albicans* cells (Fig. 2). A single macrophage could phagocytose up to 10 to 12 yeast cells, the numbers apparently depending on such factors as extent of macrophage movement, size and number of pseudopodia, and number of macrophage collisions with *C. albicans* cells. In general, a direct correlation existed between macrophage activity and the amount of phagocytosis; the more motile the cell, the greater the number of *C. albicans* cells that were engulfed. The addition of soluble *C. albicans* antigen had no visible effect on movement of the macrophage or engulfment of the yeast cells.

Phagocytosis by cells from sensitized guinea pigs. Peritoneal exudate cells were also obtained from 20 guinea pigs which had been sensitized by injection of living *C. albicans* cells. One or two weeks before removal of the peritoneal exudate cells, the animals were skin-tested with soluble *C. albicans* antigen and found to have delayed hypersensitivity. In studies in vitro on media without soluble antigen and without *C. albicans* cells, cell migration appeared normal. When killed *C. albicans* cells were allowed to spread on the agar surface and peritoneal cells were inoculated, macrophage migration was slightly slower than that observed with peritoneal exudate

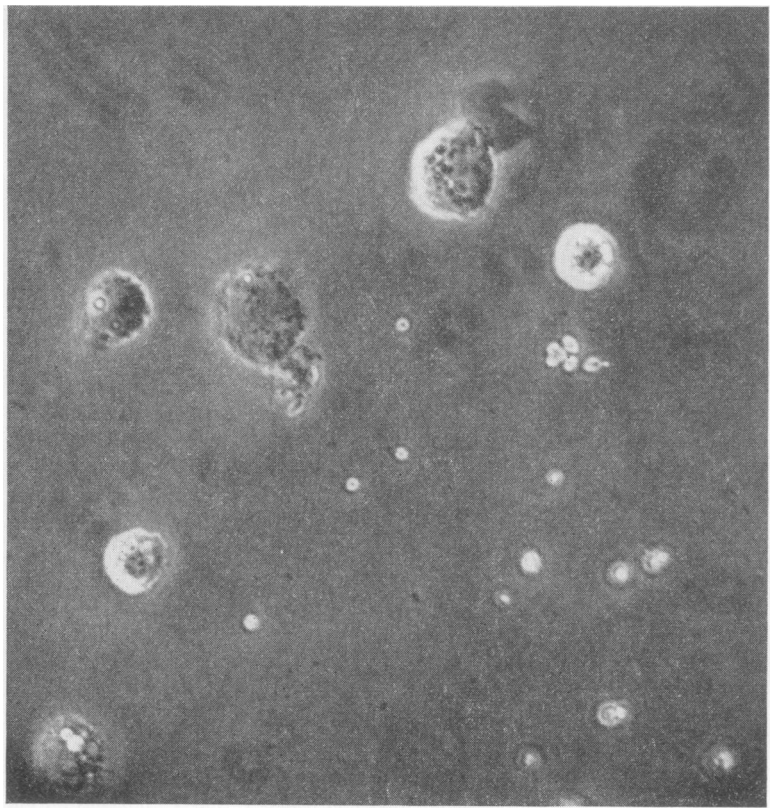

FIG. 3. *Phagocytosis of C. albicans by macrophages from sensitized guinea pigs in the presence of antigen. Note tendency of cells to become rounded, paucity of phagocytosed C. albicans, and presence of C. albicans extracellularly on agar surface.* × *700.*

cells from normal animals. This decrease in motility was paralleled by a decrease in phagocytosis.

When soluble antigen of *C. albicans* was included in the agar, the effect was striking. In comparison with sensitized cells in the absence of antigen, the migration in the presence of antigen was reduced by 50 to 70% when cell migration was measured on agar in Falcon flasks at 24 hr postinoculation. At this time, macrophage viability, as determined with 1:2,000 neutral red in the agar medium, was found to be 95 to 100% in both normal and sensitized cells in the presence of antigen. Thus, although migration was inhibited, the cells remained alive. Changes in macrophage activity were also paralleled by changes in the *p*H of the agar medium where phenol red served as an indicator. When cell activity was normal, the medium tended to become acidic within 24 hr; when activity was inhibited, the medium maintained its initial *p*H. For the first 2 to 4 hr after implantation of the peritoneal cells, migration and phagocytosis by some of the peritoneal macrophages seemed normal. Fewer cells, however, were migrating and phagocytosing. Subsequently, the macrophages appeared to lose their ability to form large, active pseudopodia and became relatively immobile, although cytoplasmic flow was still visible. They tended to round up and to adhere to one another and to nearby lymphocytes. The only cells that could phagocytose *C. albicans* at all were those that still formed some pseudopodia and thus had some motility. The fewer the number and the smaller the size of the pseudopodia, the less able was the macrophage to carry out phagocytosis (Fig. 3). The number of unphagocytosed *C. albicans* cells was very high. Often, yeast cells were observed in close contact with a macrophage; yet, engulfment did not follow. Pseudopodia would be formed in close proximity to a *C. albicans* cell but would not phagocytose the yeast. Ultimately, many of the macrophages became completely immotile, without apparent cytoplasmic activity. The agar surface thus consisted of small clusters of nonmigrating, adherent mononuclear cells, surrounded by many unphagocytosed *C. albicans*.

DISCUSSION

Demonstration in vitro of a process that occurs in an intact animal isolates only certain effects. Such a process is significant when it can be shown to exist in vivo as part of a sequence. The isolation in vitro of peritoneal exudate cells from sensitized and normal animals permits the study of some of the mechanisms associated with delayed hypersensitivity and cellular immunity, such as interaction of cells, release of mediators, and sequence of immunologic responses.

Examination in vitro of cells from guinea pigs with delayed hypersensitivity revealed that macrophages moved actively on agar in the absence of soluble antigen and at the same time carried out active phagocytosis of cells of *C. albicans*. When such cells from sensitized animals were implanted on antigen-containing agar, both their migration and their phagocytosis were inhibited. The movement of pseudopodia, which seems so important in the engulfment of particles, was greatly reduced both in magnitude and in rate. At the moment macrophage activity was reduced, the expenditure of energy was probably affected. Whether this decrease in energy output and the inhibition of phagocytosis were associated with decreased adenosine triphosphate activity and with decreased availability of chemical energy is not yet known. In addition, the macrophages and lymphocytes tended to aggregate. All these effects occurred within 24 hr after exposure of the mononuclear cells to antigen. The animals that produced the sensitized cells and exhibited delayed hypersensitivity also had an enhanced cellular resistance, since the tissues of such guinea pigs yielded fewer *C. albicans* than did the tissues of normal animals (9).

A decreased number of pathogens in phagocytosing macrophages may suggest either decreased phagocytosis or increased phagocytosis accompanied by increased killing. Cinemicrographic studies with *C. albicans* have demonstrated that, when the migration of sensitized macrophages is inhibited by the production of inhibitory factors from lymphocytes, engulfment of the *C. albicans* cells is simultaneously reduced.

Migration or inhibition of migration of individual macrophages is readily recognizible on an agar surface. Whether such migration is the primary cause of movement out of a capillary tube has not been proved, as pressure changes induced by alterations in metabolic rate, membrane permeability, or membrane stickiness may also cause the cells to emerge or remain in the capillary tube.

Inhibition of macrophage "spreading" has been observed in mice infected with *Listeria monocytogenes* and in guinea pigs sensitized with BCG (2). Mouse peritoneal exudate cells were removed, counted, and incubated in a moist chamber at 37 C for 30 min. In the presence of soluble *L. monocytogenes* antigen, the macrophages showed a markedly reduced amount of spreading, i.e., 38% with antigen as compared with 93% in normal mice under similar conditions. A similar decrease in spreading ability was recorded in experiments with guinea pig peritoneal exudate cells. Herein, after 30 min of exposure to tuberculin, only 20% of macrophages from tuberculin-sensitive guinea pigs exhibited spreading ability, whereas in the presence of ovalbumin as antigen, inhibition did not occur. Inhibition of spreading and inhibition of migration were believed to develop under the same conditions.

Cell counts have also been made on peritoneal exudate cells of normal versus sensitized guinea pigs (14). Since some of the animals had been sensitized to the tubercle bacillus, the culture medium contained varying concentrations of either old tuberculin, "purified protein derivative," or glycerin. Antigen in appropriate concentrations caused an early fall in total cell counts. Surviving cells reached a maximum decline in 18 to 24 hr and then started to proliferate more extensively than the controls. By 72 to 90 hr after exposure to antigen, cells from sensitized animals were proliferating more extensively than similar cells in the absence of antigen. The decreased proliferation at 24 hr may be an expression in vitro similar to inhibition of migration after the same exposure in time to antigen.

Activation of normal guinea pig peritoneal cells has also been demonstrated with soluble supernatant fluid from sensitized lymphocytes which had been exposed to specific antigen (6). This supernatant fluid inhibited the migration of normal macrophages from capillary tubes. In addition, when such cells were exposed to the supernatant fluid or a fraction thereof for a period of 72 hr, the following phenomena were observed: increased adherence on glass surfaces, increased oxidation of glucose-carbon-1, and increased phagocytosis of starch particles and mycobacteria in guinea pigs sensitized to a heterologous hapten-protein conjugate. Thus, normal macrophages apparently can be inhibited on exposure for 24 hr to soluble lymphocyte supernatant fluid containing migration-inhibitory factor but are activated after exposure for 72 hr.

It may be suggested therefore that the enhanced resistance associated with delayed hypersensitivity is biphasic. In this regard, the early expression of such resistance in vivo may involve "walling off" of the invading organisms or foreign

cells; the inhibition of migration and reduction in phagocytosis would thus be beneficial to the host in that distribution of the invader would be limited. Subsequently, the host phagocytes assume an activated, destructive role. Such a state is indicated by changes in macrophage activity in vivo at certain stages of infection, such as increase in mitotic rate, increase in cell lysosomes and vesicles, "spreading" on glass, and enhanced capacity for phagocytosis (5). The rounding up and immobilization of a macrophage indicate reduction in energy consumption, as "spreading" of a macrophage indicates an increase in energy consumption (7).

Antibody may be present in the suspensions of peritoneal exudate cells, since they contain lymphocytes from previously sensitized guinea pigs. Complexes may therefore have formed when antigen was added to the cell suspension to induce the production of migration-inhibitory substances. Such antigen-antibody complexes have the capacity to increase the extent of macrophage spreading (7) or to enhance the respiratory activity of macrophages (13). Whether such complexes by themselves can inhibit the migration of normal peritoneal macrophages on an agar surface is not known. Since the migration-inhibitory effect seems to lessen after 72 to 96 hr, and since the macrophages migrate out onto fresh areas of possible complex-containing agar, it seems unlikely that antigen-antibody complexes by themselves inhibit macrophage migration over an extended period of time.

The lymphocyte-macrophage interactions produce a dynamic series of factors and effects. However, many unanswered questions remain. The time at which these factors become active after antigen stimulation and the sequence in which they are formed are unknown. Whether one or several types of lymphocytes secrete these factors and whether the effect is on one or more types or stages of macrophages also are not known. The threshold for release of the various factors may be the same for a given antigen, or the different factors may be released at different concentrations of antigen. Those cells that have released the factors or reacted to them may also be desensitized to such a degree that a recovery period is required before further activity appears. Studies on this subject are now being conducted.

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