INFECTION AND IMMUNITY, May 1976, p. 1442-1448

Vol. 13, No. 5
Printed in U.S.A.

Different Effects of Phytohemagglutinin-Activated Lymphocytes and Their Culture Supernatants on Macrophage Function

MASAYUKI ANDO,* MORITAKA SUGA, KIYOSHI SHIMA, MINEHARU SUGIMOTO, SADANOBU HIGUCHI, TOMIYASU TSUDA, AND HARUHIKO TOKUOMI

The First Department of Internal Medicine, Kumamoto University Medical School, Kumamoto 860, Japan

Received for publication 25 November 1975

When rabbit peritoneal exudate cells were incubated for 24 and 48 h with phytohemagglutinin-activated lymphocytes or their culture supernatants, two times as many cells remained adherent to culture slides as in the controls. More spreading cells were found among the adherent cells in the stimulated cultures. Eighty percent of spreading cells that were induced by supernatants were negative or faintly positive for β-galactosidase. On the other hand, half of the spreading cells induced by activated lymphocytes were positive (1+ to 4+) for β-galactosidase. Enhanced reduction of nitroblue tetrazolium was found in the spreading cells that were stimulated by either phytohemagglutinin-activated lymphocytes and their supernatants. Under similar conditions, unstimulated peritoneal cells showed less marked activation. These findings show that macrophages can appear morphologically activated and yet not be enzymatically activated by lymphokines. Possible mechanisms of direct interaction of activated lymphocytes and macrophages are discussed.

Macrophages, entering from the bloodstream into dermal BCG lesions or tuberculin reactions of tuberculin-positive rabbits, develop increased β-galactosidase activity. Immunological activation of macrophages, as monitored by their β-galactosidase activity in vivo, was clearly associated with the presence of delayed hypersensitivity (1, 2, 9).

It is thought that macrophage activation is induced mainly by lymphokines, released by antigen-stimulated lymphocytes. In fact, there are many reports that lymphokines affect macrophages and cause their morphological and functional alterations (18, 20, 26). Whether lymphokines directly induce elevation in the hydrolytic enzyme activity of macrophages is not certain. According to some reports, lymphokines do not affect the activity of macrophage hydrolytic enzymes (19; H. G. Remold and A. Mednis, Fed. Proc., p. 753, 1972).

This report shows that lymphokines from phytohemagglutinin (PHA)-activated lymphocytes do not affect the level of the hydrolytic enzyme β-galactosidase in macrophages and that intact PHA-activated lymphocytes do affect it. In other words, lymphokines and activated lymphocytes had different effects on the activation of macrophages in vitro.

MATERIALS AND METHODS

Animals. Female New Zealand white rabbits, weighing 2 to 3 kg, were used in all experiments.

Culture media. RPMI 1640 medium (GIBCO, Grand Island, N.Y.) supplemented with 1% L-glutamine (200 mM) and 10% fetal calf serum, heat-inactivated for 30 min at 56 C, was used for all experiments. Penicillin (100 U/ml) and streptomycin (100 μg/ml) were added.

Harvest of macrophages. Animals were exsanguinated by severing the carotid artery with a sharp blade. Peritoneal cells (PC) were harvested from normal rabbits with 200 ml of heparinized (5 U/ml) Hanks balanced salt solution (HBSS). Peritoneal exudate cells (PEC) were obtained 4 days after the intraperitoneal injection of 100 ml of 0.4% glycogen in saline. The two types of peritoneal cells were washed three times with HBSS and resuspended in culture medium. PC contained 80% macrophages, 15% lymphocytes, and 5% polymorphonuclear leukocytes. PEC contained 88% macrophages, 3% lymphocytes, and 9% polymorphonuclear leukocytes.

Preparation of macrophage monolayers. For macrophage cultures, the cells, at a concentration of 1.0×10^6/chamber, were allowed to settle on four chamber tissue culture slides (Lab-Tek Products, Westmont, Ill.). Two hours after incubation at 37 C in 5% CO_2 and 95% humidified air, the chambers were washed five times with HBSS to remove nonadherent cells, and fresh culture medium was added.

Harvest of lymph node cells. After the macrophages were harvested, mesenteric lymph nodes were obtained. The capsular membrane of lymph nodes was gently removed with a corneal plantation pincette, and the cells were obtained by shaking the nodes in cold HBSS. The cells were passed through a stainless-steel screen (30 mesh) to remove tissue

fragments, washed three times in cold HBSS, and resuspended in culture medium. Ninety-two percent of the cells were lymphocytes, 87 to 92% of which were viable by trypan blue exclusion.

Preparation of PHA-activated lymphocytes and their culture supernatants. Lymph node lymphocytes were incubated in the presence or absence of 6 μl of PHA-P per ml (Difco Laboratories, Detroit, Mich.) in plastic tubes (no. 2001, Falcon Plastics, Los Angeles, Calif.). Viable lymphocytes, 10^7, were suspended in 5 ml of culture medium. After incubation for 18 h at 37 C in 5% CO_2 and 95% humified air, the culture tubes were centrifuged at 200 $\times$ g for 5 min. Supernatants were discarded, and the sediments were washed three times with culture medium to remove the free PHA-P. After another incubation for 24 h, the culture tubes were centrifuged at 200 $\times$ g for 10 min. The supernatants were used as a source of lymphokines after centrifugation at 1,500 $\times$ g for 30 min. The sediments were washed twice, and used as a source of activated lymphocytes. The cells in this stage were 90% viable by trypan blue exclusion. An aliquot of the lymphocyte preparation was cultured for an additional 54 h, during which time 60% of the cells became blastoid.

Measurement of cellular adherence. Two methods were used to count the number of adherent cells at 24 and 48 h after incubation. One was to count with a hemacytometer the macrophages released by shaking the culture slides. The other was to count the number of macrophages remaining adherent around the center of the culture slide in 60 microscope fields at 1,000$\times$ magnification (143 mm^2). The population of adherent cells was confirmed to consist mostly of macrophages, as judged by phagocytic capacity of polystyrene latex particles.

Morphological classification. Macrophages were classified by morphological criteria as rounded cells, spindle cells, and spreading cells. Cells in the first two classifications were considered to be nonactivated macrophages, and cells in the last classification were considered to be activated macrophages.

Staining of β-galactosidase. The culture slides were fixed for 4 min in cold, buffered 1.25% glutaraldehyde, rinsed in saline, and incubated 18 h in Pearson indolyl substrate for β-galactosidase (5-bromo-4-chloro-3-indolyl-β-D-galactoside, Cyclo Chemical Division of Travenol Laboratories, Inc., Los Angeles, Calif.) as previously reported (28).

NBT reduction by macrophages. One milliliter of nitro blue tetrazolium (NBT) solution (1 part of 0.3% NBT in saline, 1 part of 0.1 M phosphate buffer solution [pH 7.35]), which had been sterilized through a 0.45-μm membrane filter (Millipore Corp.) was added to the chamber slides, and the preparations were incubated at 37 C for 20 min. The culture slides were fixed for 4 min in 1.25% glutaraldehyde and were counterstained with hematoxylin. The cells containing clumped formazan were designated 2+ positive cells, and the cells with 15 or more formazan granules were designated 1+ positive cells.

Assay for MIF. The whole-culture supernatants were assayed for magrophage inhibitory factor (MIF) activity by a modification of the method of David et al. (10). PEC from normal rabbits were induced by mineral oil. The cells were washed in HBSS and suspended to 10% by volume in culture medium. Capillary tubes were filled with the cell suspension and centrifuged. The tubes were cut just below the cell/fluid interface, and the portion containing the cells was placed in Lab-Tek chambers. They were incubated at 37 C for 36 h, and the area of migration was projected and measured by planimetry. The percentage of migration inhibition was estimated by the formula: 100 $-$ ([average area of migration with culture supernatant]/[average area of migration without culture supernatant]) $\times$ 100. Migration was considered to be inhibited and MIF to be present when the inhibition was more than 20% of the corresponding control culture.

Statistical method. Standard error of the means $\sqrt{\Sigma(X - \overline{X})^2/n(n-1)}$ and P values were calculated using the Student t method.

RESULTS

Effect of activated lymphocytes and their supernatants on PEC and PC adherence. More adherent cells were observed when PEC and PC were incubated with activated lymphocytes or their supernatants than control lymphocytes or their supernatants at 24 and 48 h (Table 1).

Average numbers of adherent PEC per unit area of chamber slides cultured with activated lymphocytes or their supernatants were twice those of controls ($P < 0.009$ and $P < 0.006$, respectively).

These differences were also confirmed by counting the nonadherent cells, which were 99% viable as determined by the exclusion of trypan blue dye and by ingestion of polystyrene latex particles (unpublished observations). Control chambers contained twice the number of non-adherent cells than chambers treated with activated lymphocytes and their supernatants ($P < 0.015$ and $P < 0.009$, respectively).

These effects were also observed with PC, but the differences were not as significant. Activated lymphocytes and their supernatants had similar effects on the adherence of PEC and PC.

Morphological alterations of PEC and PC. After incubation with activated lymphocytes or their supernatants, PEC showed remarkable morphological alterations at 24 and 48 h (Table 1). The cells were much larger and contained abundant cytoplasm and irregular membranes. Many phase-dense granules and vacuoles were seen (Fig. 1 and 2). These morphological characteristics were similar to those seen in activated macrophages described by Mackaness (16) and Dannenberg (8).

The morphological alterations induced by activated lymphocytes and their supernatants were similar. Neither preparation induced

TABLE 1. *Numbers of PEC attached to the culture slides and morphological changes after incubation with activated lymphocytes or lymphokines*[a]

Stimulants added to PEC monolayer	Incubation time (h)	Adherent PEC per unit area		Rounded and spindle cells		Spreading cells	
		Total no.	*P* values (a vs. b)	No.	*P* values (a vs. b)	No.	*P* values (a vs. b)
(a) Activated lymphocytes	24	1,195 ± 103	0.009	537 ± 73	0.462	658 ± 98	0.003
(b) Normal lymphocytes		792 ± 73		547 ± 82		245 ± 10	
(a) Activated lymphocytes	48	890 ± 67	0.004	412 ± 71	0.157	478 ± 37	0.003
(b) Normal lymphocytes		588 ± 38		329 ± 25		259 ± 35	
(a) Lymphokines	24	1,079 ± 54	0.028	551 ± 130	0.214	528 ± 60	0.005
(b) Control supernatants		664 ± 84		437 ± 35		227 ± 50	
(a) Lymphokines	48	1,029 ± 31	0.006	449 ± 54	0.039	580 ± 95	0.005
(b) Control supernatants		483 ± 72		302 ± 43		181 ± 48	

[a] Four identical experiments were carried out.

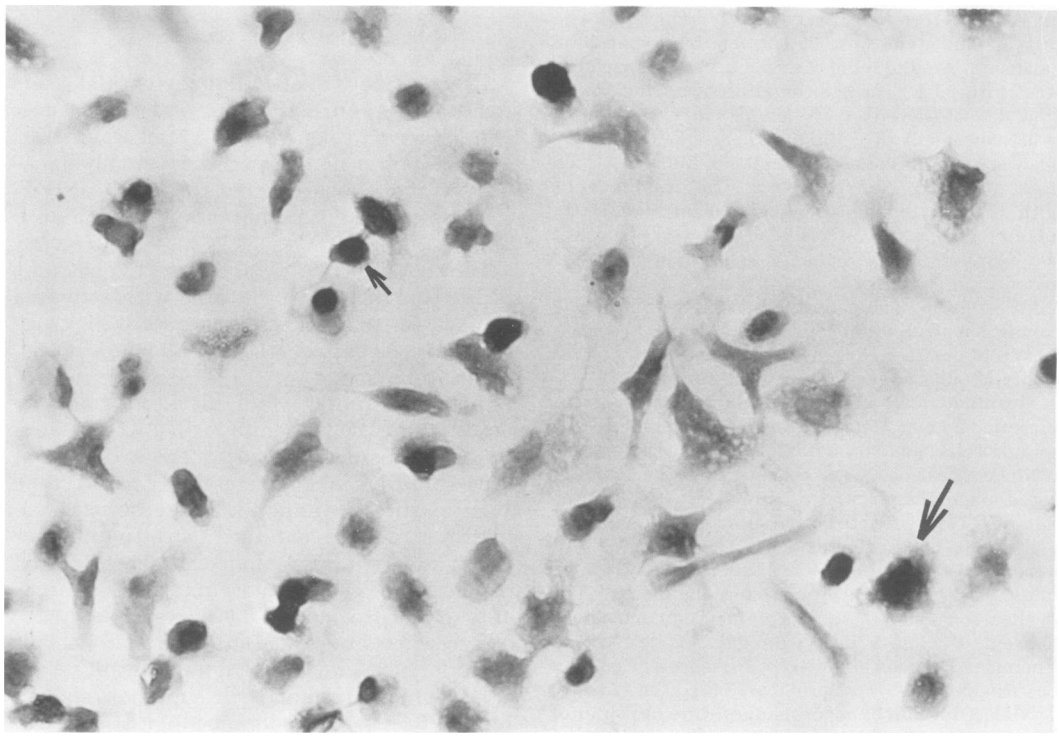

FIG. 1. *A monolayer of PEC incubated for 48 h with whole-culture supernatants from PHA-activated lymphocytes. Many of the spreading cells are negative or faintly stained for β-galactosidase. Ten of the cells (small arrow) staining more than 1+ for β-galactosidase are seen in this photograph. Only one of them shows spreading (large arrow). (5-Bromo-4-chloro-3-indolyl-β-D-galactosidase, hematoxylin and eosin; ×2,500).*

much alteration after 3, 6, and 12 h of incubation.

Both activated lymphocytes and their supernatants affected one of the morphological features of PC in vitro. The morphological alterations in PC, however, were not as pronounced as those in PEC.

β-Galactosidase activity of PEC and PC. The most interesting results were the differences in the effects of activated lymphocytes and their supernatants on the β-galactosidase activity of spreading cells. Macrophages (PEC and PC) treated with supernatants did not show increased β-galactosidase activity at 24 and 48 h in spite of remarkable alterations in their morphology (Fig. 1 and 3). On the other hand, macrophages treated with activated lymphocytes did show increased β-galactosidase ac-

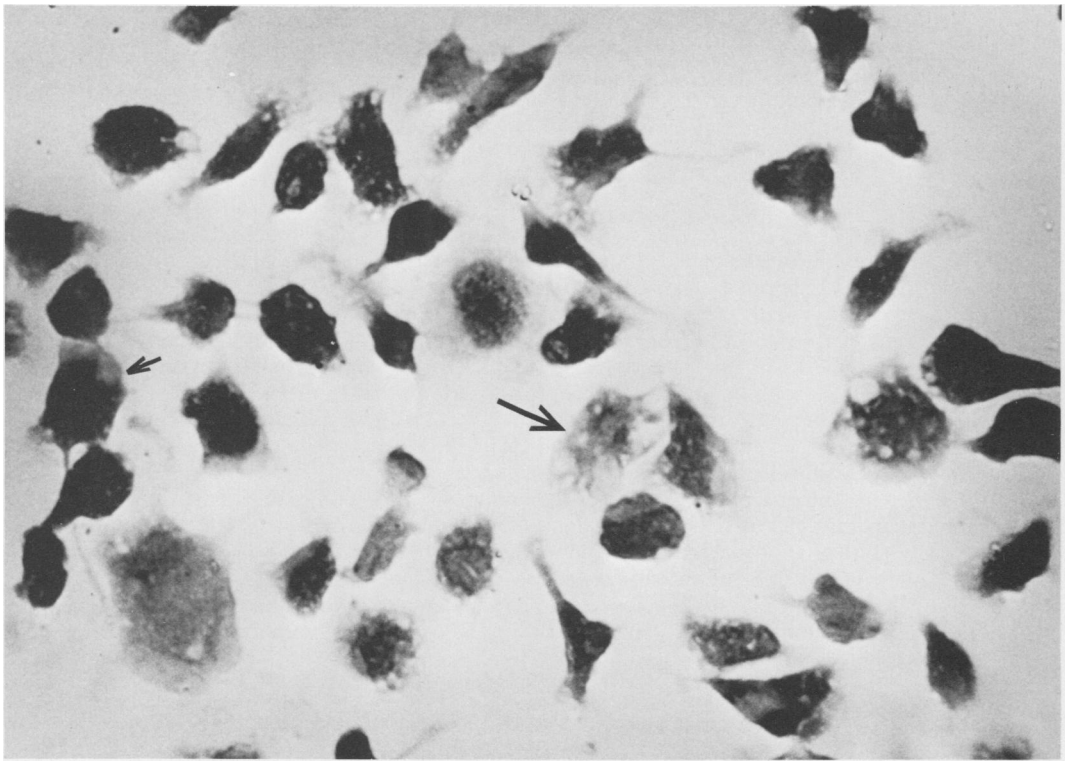

FIG. 2. *A monolayer of PEC incubated with PHA-activated lymphocytes for 24 h. In contrast with Fig. 1, most of the spreading cells are stained 1+ to 3+ for β-galactosidase. A typical 3+ cell is marked with a small arrow, and a typical negative cell is marked with a large arrow. (5-Bromo-4-chloro-3-indolyl-β-D-galactosidase, hematoxylin and eosin; ×2,500).*

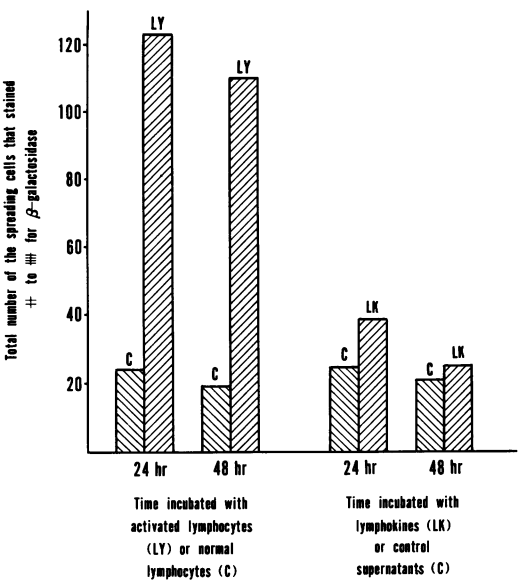

FIG. 3. *Number of spreading cells per unit area (143 mm²) that were 2+ to 4+ positive for β-galactosidase after incubation with activated lymphocytes or their whole-culture supernatants.*

tivity at 24 and 48 h ($P < 0.007$ and $P < 0.016$, respectively) (Fig. 2 and 3). Twenty-four-hour incubation with activated lymphocytes showed the greatest effect (Fig. 3).

The percentages of 1+ and 2+ to 4+ β-galactosidase-positive cells among the spreading cells induced by activated lymphocytes were 30% and 24, respectively, compared with 17 and 6% for lymphocyte supernatants. The total numbers of 2+ to 4+ β-galactosidase-positive cells per unit area in the cultures treated with activated lymphocytes were four times those found in cultures treated with supernatants at 24 and 48 h ($P < 0.009$ and $P < 0.022$, respectively). β-Galactosidase activity in rounded and spindle cells that were incubated with activated lymphocytes or their supernatants was similar to that of the controls.

NBT reduction by PEC. About 30% of the spreading cells induced by activated lymphocytes or their supernatants were weakly positive for NBT reduction, compared with less than 9% of the rounded and spindle cells. The few spreading cells found in the control macrophage monolayers treated with normal lympho-

cytes or their supernatants were also positive for NBT reduction to the same degree (Fig. 4). In other words, NBT reduction of PEC was correlated with the spreading of macrophages, regardless of the stimulus that caused this spreading. In PEC, the amount of β-galactosidase activity was not correlated with the amount of NBT reduction.

Effect of incubation time. Activated lymphocytes may release different mediators when their incubation was prolonged. Therefore, the supernatants from 48-h cultures of activated lymphocytes were evaluated. The results, however, were the same as with supernatants from 24-h cultures. MIF was present in the supernatants from 24- and 48-h cultures of activated lymphocytes. Inhibition of migration averaged 40 to 50%. Tenfold-diluted supernatants that had little effect on the morphology of macrophage monolayers did not produce migration inhibition. The control supernatants from normal lymphocytes did not inhibit the migration of macrophages. Activated lymphocytes used immediately after incubation with PHA-P for 18 h produced effects similar to activated lymphocytes used at 24 h after removal of the PHA-P.

DISCUSSION

Both activated lymphocytes and their whole-culture supernatants increased the adherence of PEC and PC to glass and caused morphological alterations to a similar degree. However, in PEC and PC showing these alterations, increased β-galactosidase activity was induced only by activated lymphocytes, and never by their supernatants. Macrophages activated by sensitized lymphocytes (incubated with antigen) are probably more effective in killing bacteria than macrophages activated by some of the lymphokines, in some instances (14, 29, 30), because the level of β-galactosidase in macrophages can be used as a measure of the ability of macrophages to destroy the tubercle bacillus, as reported previously (2, 3, 9).

The whole-culture supernatants obtained from PHA-activated lymphocytes in this experiment contained lymphokines, including MIF

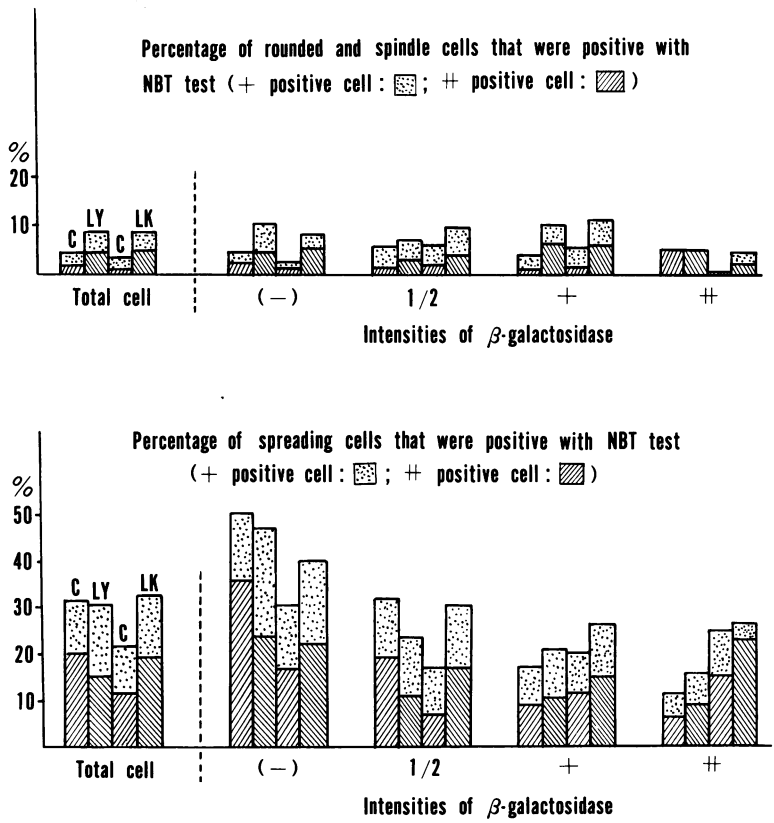

FIG. 4. *Reduction of NBT in activated and nonactivated macrophages. C, Normal lymphocytes; L, activated lymphocytes.*

and, presumably, macrophage-activating factors. The increased adherence and morphological alterations of PEC and PC after incubation with the supernatants resembled those found by Mooney and Waksman (18). They incubated rabbit PEC for 24 to 48 h with unfractionated supernatants from antigen-stimulated lymph node cells.

From the findings presented here, it appears that lymphokines were not the main factors increasing the β-galactosidase activity of macrophages, unless unstable mediators are involved. If not via lymphokines, the question arises as to how activated lymphocytes caused the increased β-galactosidase activity of macrophages.

A strong possibility is a direct membrane interaction between activated lymphocytes and macrophages. In our experiments, the culture slides were shaken for counting nonadherent cells and were rinsed with saline to remove floating cells before fixation with glutaraldehyde. Therefore, we did not constantly observe the findings of lymphocyte-macrophage interaction. Direct membrane interaction by means of uropods has been demonstrated in several experimental systems using the methods of electron microscopy (15, 27) and time-lapse phase-contrast cinemicrography (24, 25). This evidence suggests that stimulatory materials of activated lymphocytes may be acquired by macrophages through this mechanism.

The bactericidal activity of neutrophils is known to be closely associated with oxidative process of the cells; i.e., oxygen-dependent antimicrobial systems, in the presence or abscence of myeloperoxidase, play an important role in the destruction of certain microorganisms (13). On the other hand, macrophages are activated by lymphokines and shown higher consumption of oxygen and hexose monophosphate oxidation in the culture (20, 21). Therefore, it is of interest to investigate whether the macrophages in this study are activated or not in respect of oxidative metabolism.

NBT tests were performed to observe indirectly the activities of oxidative metabolism in each cell level. About one-third of the morphologically activated PEC that were stimulated by activated lymphocytes or lymphokines were positive for the NBT test, as compared with less than 10% of the nonactivated PEC (Fig. 4). Little is known about the mechanisms of reduction of NBT in macrophages. In neutrophils, associated with NBT reduction is a general increase in oxidative metabolism, characterized by increases in oxygen consumption (4), hexose monophosphate shunt activity (4, 23), and production of hydrogen peroxide (5, 23) and the superoxide anion (6, 7).

Whether or not the mechanisms of NBT reduction similar to those found in neutrophils exist in macrophages is not clear. But NBT reduction may reflect the enhanced oxidative metabolism of macrophages, since spreading cells were more positive for the NBT test than rounded or spindle cells. If so, the macrophages activated by lymphokines (or activated lymphocytes) may kill certain bacilli through the process of oxygen-dependent antimicrobial systems. The amount of NBT reduction in activated PEC was weak, though, in contrast with cultured alveolar macrophages or neutrophils (unpublished observations). This phenomenon may be related to the evidence that the macrophages activated by lymphokines exhibit enhanced bacteriostasis (11, 12, 22). Further work is required to show whether there are differences between the antimicrobial systems in the macrophages that were activated by sensitized lymphocytes and those found in the macrophages activated by lymphokines, because no correlation between the amounts of NBT reduction and β-galactosidase activity was found in activated macrophages.

ACKNOWLEDGMENT

This work was supported partially by a grant from the Japanese Ministry of Education.

LITERATURE CITED

1. **Ando, M.** 1973. Macrophage activation in tuberculin reactions of rabbits with primary BCG infection and reinfection. J. Reticuloendothel. Soc. **14**:132–145.
2. **Ando, M., and A. M. Dannenberg, Jr.** 1972. Macrophage accumulation, division, maturation, and digestive and microbicidal capacities in tuberculous lesions. IV. Macrophage turnover, lysosomal enzymes, and division in healing lesions. Lab. Invest. **27**:466–472.
3. **Ando, M., A. M. Dannenberg, Jr., and K. Shima.** 1972. Macrophage accumulation, division, maturation, and digestive and microbicidal capacities in tuberculous lesions. II. Rate at which mononuclear cells enter and divide in primary BCG lesions and those of reinfection. J. Immunol. **109**:8–19.
4. **Baehner, R. L., and D. G. Nathan.** 1967. Leukocyte oxidase: defective activity in chronic granulomatous disease. Science **155**:835–836.
5. **Baehner, R. L., D. G. Nathan, and M. L. Karnovsky.** 1970. Correction of metabolic deficiencies in the leukocytes of patients with chronic granulomatous disease. J. Clin. Invest. **49**:865–870.
6. **Barior, B. M., R. S. Kipnes, and J. T. Curnutte.** 1973. Biological defense mechanisms. The production by leukocytes of superoxide, a potential bactericidal agent. J. Clin. Invest. **52**:741–744.
7. **Curnutte, J. T., D. M. Whitten, and B. M. Barior.** 1974. Defective superoxide production by granulocytes from patients with chronic granulomatous disease. N. Engl. J. Med. **290**:593–597.
8. **Dannenberg, A. M., Jr.** 1968. Cellular hypersensitivity and cellular immunity in the pathogenesis of tuberculosis. Specificity, systemic and local nature, and

associated macrophage enzymes. Bacteriol. Rev. 32:85-102.
9. **Dannenberg, A. M., Jr., M. Ando, and K. Shima.** 1972. Macrophage accumulation, division, maturation, and digestive and microbicidal capacities in tuberculous lesions. III. The turnover of macrophages and its relating to their activation and antimicrobial immunity in primary BCG lesions and those of reinfection. J. Immunol. 109:1109-1121.
10. **David, J. R., S. Al-Askari, H. S. Lawrence, and L. Thomas.** 1964. Delayed hypersensitivity in vitro. 1. The specificity of inhibition of cell migration by antigens. J. Immunol. 93:264-273.
11. **Fowles, R. E., I. M. Fajardo, J. L. Leibowitch, and J. R. David.** 1973. The enhancement of macrophage bacteriostasis by products of activated lymphocytes. J. Exp. Med. 138:952-964.
12. **Godal, T., R. J. W. Rees, and J. O. Lamvik.** 1971. Lymphocyte-mediated modification of blood-derived macrophage function in vitro; inhibition of growth of intracellular mycobacteria with lymphokines. Clin. Exp. Immunol. 8:625-637.
13. **Klebanoff, S. J.** 1975. Antimicrobial mechanisms in neutrophilic polymorphonuclear leukocytes. Semin. Hematol. 12:117-142.
14. **Leibowitch, J., and J. R. David.** 1973. Lymphocyte-macrophage interaction in resistance to Listeria monocytogenes. Ann. Immunol. 124C:30-51.
15. **Lipsky, P. E., and A. S. Rosenthal.** 1973. Macrophage-lymphocyte interaction. I. Characteristics of the antigen-independent-binding of guinea pig thymocytes and lymphocytes to syngeneic macrophages. J. Exp. Med. 138:900-924.
16. **Mackaness, G. B.** 1970. Cellular immunity, p. 461-475. *In* R. Van-Furth (ed.), Mononuclear phagocytes. F. A. Davis Co., Philadelphia, Pa.
17. **Mandell, G. L., W. Rubin, and E. W. Hook.** 1970. The effect of an NADH oxidase inhibitor (hydrocortisone) on polymorphonuclear leukocyte bactericidal activity. J. Clin. Invest. 49:1381-1388.
18. **Mooney, J. J., and B. H. Waksman.** 1970. Activation of normal rabbit macrophage monolayers by supernatants of antigen-stimulated lymphocytes. J. Immunol. 105:1138-1145.
19. **Nath, I., L. W. Poulter, and J. L. Turk.** 1973. Effect of lymphocyte mediators on macrophages in vitro. A correlation of morphological and cytochemical changes. Clin. Exp. Immunol. 13:455-466.
20. **Nathan, C. F., M. L. Karnovsky, and J. R. David.** 1971. Alterations of macrophage functions by mediators from lymphocytes. J. Exp. Med. 133:1356-1376.
21. **Nathan, C. F., H. G. Remold, and J. R. David.** 1973. Characterization of a lymphocyte factor which alters macrophage functions. J. Exp. Med. 137:275-290.
22. **Patterson, R. J., and G. P. Youmans.** 1970. Demonstration in tissue culture of lymphocyte mediated immunity to tuberculosis. Infect. Immun. 1:600-603.
23. **Reed, P. W.** 1969. Glutathione and the hexose monophosphate shunt in phagocytizing and hydrogen peroxide-treated rat leukocytes. J. Biol. Chem. 244:2459-2464.
24. **Salvin, S. B., S. Sell, and J. Nishio.** 1971. Activity in vitro of lymphocytes and macrophages in delayed hypersensitivity. J. Immunol. 107:655-662.
25. **Sharp, J. A., and R. G. Burwell.** 1960. Interaction (periolesis) of macrophages and lymphocytes after skin homografting or challenge with soluble antigens. Nature (London) 188:474-475.
26. **Schmidt, M. E., S. D. Douglas, and A. D. Rubin.** 1973. Human monocyte activation by supernatants from concanavalin A (Con A) stimulated lymphocytes. Cell. Immunol. 9:45-59.
27. **Schoenberg, M. D., V. R. Mumaw, R. D. Moore, and A. S. Weisberger.** 1974. Cytoplasmic interaction between macrophages and lymphocytic cells in antibody synthesis. Science 143:964-965.
28. **Shima, K., A. M. Dannenberg, Jr., M. Ando, S. Chandrasekhar, J. A. Selzicki, and J. I. Fabrikant.** 1972. Macrophage accumulation, division, maturation, and digestive and microbicidal capacities in tuberculous lesions. I. Studies involving their incorporation of tritiated thymidine and their content of lysosomal enzymes and bacilli. Am. J. Pathol. 67:159-180.
29. **Simon, H. B., and J. N. Sheagren.** 1972. Enhancement of macrophage bactericidal capacity by antigenically stimulated immune lymphocytes. Cell. Immunol. 4:163-174.
30. **Simon, H. B., and J. N. Sheagren.** 1972. Migration inhibitory factor and macrophage bactericidal function. Infect. Immun. 6:101-103.