INFECTION AND IMMUNITY, June 1970, p. 600-603
Vol. 1, No. 6
Printed in U.S.A.
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NOTES

Demonstration in Tissue Culture of Lymphocyte-Mediated Immunity to Tuberculosis

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Received for publication 26 February 1970

We have shown that splenic lymphocytes from mice immunized with viable attenuated mycobacterial cells (H37Ra) or ribonucleic acid prepared from these cells will bring about inhibition of multiplication of virulent tubercle bacilli (H37Rv) within normal mouse peritoneal macrophages in tissue culture. Apparently these lymphocytes, when stimulated with viable virulent tubercle bacilli, elaborate a filterable substance(s) which is responsible for this intracellular growth inhibitory effect.

Acquired cellular immunity to infection with the facultative intracellular parasite *Mycobacterium tuberculosis* is defined as the ability of immune animals to inhibit intracellular mycobacterial proliferation. At a cellular level, in vitro assays have indicated that the mononuclear phagocytes from immunized animals are the cells responsible for this intracellular growth restriction (2, 7, 8, 14, 18). In most in vitro experiments, low concentrations of streptomycin have been incorporated into the culture medium to prohibit extracellular, but presumably not intracellular, mycobacterial multiplication (1, 2, 7-10, 16-18). However, we have shown that low concentrations of streptomycin do inhibit intracellular proliferation of virulent tubercle bacilli within both normal and "immune" noninduced mouse peritoneal macrophages (12). In the presence of the antibiotic, the reduction in the number of intracellular mycobacteria is greater within immune macrophages than within normal macrophages. The inhibitory effect of streptomycin on intracellular mycobacterial growth is directly proportional to the concentration of the antibiotic employed in the medium. Concomitant with this reduction in bacillary multiplication within both normal and immune macrophages, survival of these cells was significantly enhanced.

Without streptomycin in the medium, virulent tubercle bacilli proliferate rapidly and at equal rates in the two cell types with a resultant logarithmic destruction of the macrophages. The only difference between normal and immune macrophages is the time of onset of macrophage death. The destruction of immune macrophages is delayed about 5 days, but then exponential destruction parallels that observed with normal macrophages. Others have noted the increased resistance to destruction of macrophages from immunized animals (3, 4). From these data, we concluded that the immune macrophage is not the sole cell responsible for acquired immunity to intracellular infection.

The finding by Gaugas and Rees (5) that antilymphocyte serum increased the susceptibility of immunized animals to tuberculous infection, and the report by Mackaness (11) that immune splenic lymphocytes would passively transfer immunity to *Listeria monocytogenes* infection suggested that lymphocytes from immunized animals might play a significant role in acquired cellular resistance to infection with facultative intracellular parasites.

The macrophage is the predominate cell which ingests tubercle bacilli in an infected animal. Therefore, we investigated the effect of adding splenic lymphocytes from nonimmunized mice or mice immunized with the attenuated H37Ra strain of *M. tuberculosis* to macrophage cultures infected with virulent mycobacteria. The macrophage-tubercle bacillus in vitro culture technique has been described in detail elsewhere (12). Briefly, noninduced mouse peritoneal macrophages were cultured on cover slips in petri dishes and infected with the virulent H37Rv strain of *M. tuberculosis*. On the day of infection, and 3 and 6 days later, splenic lymphocytes from normal mice or mice immunized 4 to 6 weeks

previously with the attenuated H37Ra strain of *M. tuberculosis* were added to each petri dish. The numbers of intracellular H37Rv cells within 100 macrophages was determined by direct count at days 0, 3, 6, and 9. To avoid adding large numbers of splenic macrophages to the culture, spleen cell suspensions were incubated for 1 hr at 37 C on glass-wool columns (13), and the lymphoid cells were eluted with saline (0.85% NaCl). The cells obtained in this manner consisted of 90 to 95% lymphocytes and 5 to 10% macrophages and polymorphonuclear cells, as judged by the appearance of stained preparations.

A typical experiment is shown in Fig. 1. It is clear that the addition of splenic lymphocytes from H37Ra-immunized mice resulted in a marked reduction in the intracellular numbers of H37Rv cells. In this experiment, lymphocytes from nonimmunized mice had no effect on the intracellular growth of the virulent mycobacteria. From this experiment and others, it was also determined that enumeration of intracellular mycobacteria at day 9 after infection resulted in the most reproducible and significant data.

In additional experiments, various numbers of lymphocytes were added to infected normal macrophages to determine the dose-response relationship. Figure 2 shows the results of one such experiment. The addition of 5×10^7 lymphocytes to each petri dish resulted in the greatest reduction of intracellular bacterial numbers when comparing immune lymphocyte-treated cultures with normal lymphocyte-treated cultures. The addition of 10^8 or more immune lymphocytes caused a reduction in macrophage survival. This reduced macrophage survival most probably resulted from the lymphotoxin-mediated target cell destruction described by other investigators (6, 15). In all experiments, immune lymphocytes always caused a much greater reduction in intracellular mycobacterial counts than a corresponding number of normal lymphocytes (Table 1). We previously showed that "activation" of macrophages by induction of a peritoneal exudate with glycogen enabled both normal and immune macrophages to increase their capacity, reducing intracellular mycobacterial counts 35 to 50% compared to the numbers of virulent tubercle bacilli within noninduced (nonactivated) peritoneal macrophages (12). Thus, the reduction of intracellular mycobacteria observed when normal lymphocytes were added to infected macrophages most likely was due to nonspecific macrophage activation.

Similar results have been obtained when immune macrophages were infected with H37Rv cells and normal or immune lymphocytes were added.

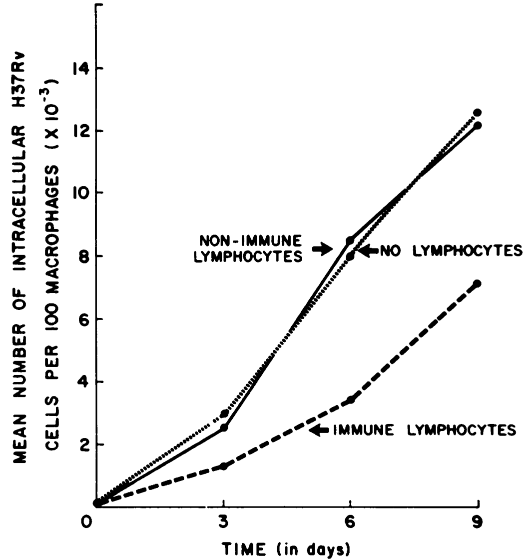

FIG. 1. *Growth of M. tuberculosis (H37Rv) within normal macrophages exposed to splenic lymphocytes from immunized and nonimmunized mice.*

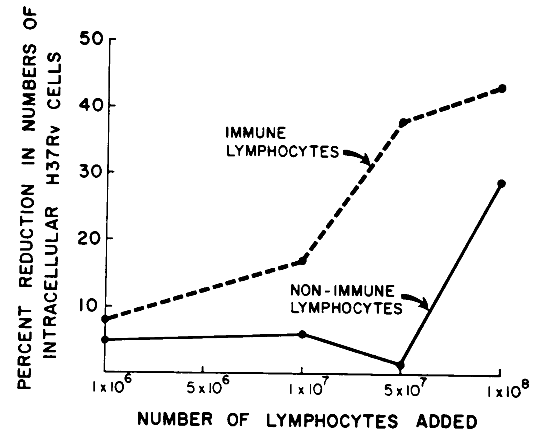

FIG. 2. *Per cent reduction of M. tuberculosis (H37Rv) within normal macrophages exposed to splenic lymphocytes from immunized and nonimmunized mice.*

To further investigate this lymphocyte-macrophage interaction, experiments were designed to determine whether results from in vitro studies correlated with the results obtained from in vivo experiments. One of us (G. P. Y.) proposed a "multiple response" theory of immunity to tuberculosis (20) composed of (i) a nonspecific activation of the reticuloendothelial system by heat-stable components of the tubercle bacillus,

TABLE 1. *Per cent reduction in the numbers of H37Rv cells within normal macrophages when treated with splenic lymphocytes from nonimmunized or immunized mice*

Expt	No. of lymphocytes[a]	Per cent reduction of		
		Nonimmune lymphocytes versus control[b]	Immune lymphocytes versus control[b]	Immune lymphocytes versus nonimmune lymphocytes[c]
1	10^7	24	26[d]	3
	10^8	54[d]	74[d]	43
2	10^7	Not done	13	
	5×10^7	6	39[d]	34[e]
3	10^6	5	8	3
	10^7	6	17	12
	5×10^7	1	38[d]	41[e]
	10^8	29	43[d]	20

[a] Number of splenic lymphocytes added at day of macrophage infection and at 3 and 6 days after infection.

[b] Macrophages not treated with lymphocytes, i.e., control.

[c] Per cent reduction calculated from numbers of H37Rv cells in macrophages treated with immune or nonimmune lymphocytes.

[d] $P < 0.05$ when comparing the numbers of H37Rv cells 9 days after infection within macrophages treated with nonimmune or immune lymphocytes versus macrophages not treated with lymphocytes.

[e] $P < 0.05$ when comparing the numbers of H37Rv cells 9 days after infection within macrophages treated with immune versus nonimmune lymphocytes.

and (ii) a more "specific" immune mechanism stimulated by a heat-labile component of viable bacilli which has been characterized as a mycobacterial ribonucleic acid (RNA) fraction (21). Accordingly, groups of mice were immunized with either 1.0 mg (moist weight) of viable H37Ra cells, 1.0 mg (moist weight) of heat-killed (autoclaved) H37Ra cells, or 50 μg of RNA from the same cells. Purified splenic lymphocytes from these and nonimmunized mice were used to treat normal H37Rv-infected macrophages (Table 2). As can be seen, lymphocytes from animals immunized with heat-killed H37Ra cells caused a slight reduction in the numbers of intracellular H37Rv cells, and this reduction paralleled the reduction obtained with an equal number of nonimmunized lymphocytes. However, lymphocytes from animals immunized with viable H37Ra cells or an equivalent amount of RNA produced

TABLE 2. *Per cent reduction in the numbers of H37Rv cells within macrophages treated with lymphocytes from immunized animals and the survival of comparably immunized animals after challenge with virulent tubercle bacilli*

No. of lymphocytes[a]	Source of lymphocytes			
	Nonimmunized mice	Heat-killed H37Ra-immunized mice	Viable H37Ra-immunized mice	RNA-immunized mice
10^7	0	17	Not done	40[b]
5×10^7	26	20	55[b]	70[b]
10^8	36[b]	27	Not done	72[b]
In vivo challenge[c]	23	30	100	79

[a] Indicated number of splenic lymphocytes added at day of macrophage infection and at 3 and 6 days after infection.

[b] $P < 0.05$ when comparing the numbers of H37Rv cells 9 days after infection within macrophages treated with lymphocytes versus macrophages not treated with lymphocytes. $P < 0.05$ when comparing the numbers of H37Rv cells 9 days after infection within macrophages treated either with lymphocytes from RNA-immunized or viable H37Ra immunized mice versus macrophages treated with lymphocytes from either heat-killed H37Ra-immunized or nonimmunized mice.

[c] Percentage of comparably immunized mice that survived more than 30 days after intravenous challenge with virulent H37Rv cells (19).

TABLE 3. *Per cent reduction in the numbers of H37Rv cells within macrophages treated with supernatants obtained from nonimmune or immune splenic lymphocytes*

Source of supernatant fraction[a]	Stimulation with H37Rv cells	Per cent reduction
Nonimmune lymphocytes	No	39[b]
	Yes	32[b]
Immune lymphocytes[c]	No	35[b]
	Yes	58[d]

[a] Supernatant fraction from 5×10^7 lymphocytes added at day of macrophage infection and at 3 and 6 days after infection.

[b] $P < 0.05$ when comparing the numbers of H37Rv cells 9 days after infection within macrophages treated with supernatant fractions versus macrophages not treated with supernatant fluid.

[c] Mice immunized with viable H37Ra cells.

[d] $P < 0.05$ when compared to the other three groups treated with supernatant fractions and to the control group not treated with lymphocyte supernatant fractions.

a significant reduction in the numbers of intracellular mycobacteria. The results obtained by challenging comparably immunized mice with virulent H37Rv cells and determining their immunity as judged by survival time (19) are also shown. It is apparent that the in vitro results correlated with in vivo results.

To test the possibility that stimulated lymphocytes produced and liberated a substance(s) that inhibited intracellular bacterial multiplication, lymphocytes were cultured in vitro, with or without stimulation by H37Rv cells, for 3 days. Cell-free supernatant fractions were obtained by centrifugation and membrane filtration (membrane filter from Millipore Corp., Bedford, Mass.). These supernatant fractions were then added to H37Rv-infected normal macrophages at days 0, 3, and 6, and the numbers of intracellular H37Rv cells were determined by direct count (Table 3).

Supernatant fractions obtained from H37Ra-immunized lymphocytes stimulated with H37Rv cells produced a marked reduction in the numbers of intracellular H37Rv cells. Supernatant fractions from nonimmune lymphocytes also caused a slight reduction apparently brought about by nonspecific macrophage activation, since supernatant fractions from either stimulated or nonstimulated nonimmune lymphocytes produced reductions similar to that observed with the supernatant fraction from immune nonstimulated lymphocytes.

The results presented in this paper represent the first direct in vitro assay of acquired cellular resistance to tuberculous infection and may provide the basis for the determination of the nature of the mechanism of acquired immunity to infection with facultative intracellular parasites.

This investigation was supported by Public Health Service research grant AI-01636 from the National Institute of Allergy and Infectious Diseases and a grant from the Tuberculosis Institute of Chicago and Cook County. R. J. P. was a predoctoral trainee supported by Microbiology Training grant 5T1-GM-724 from the National Institute of General Medical Sciences.

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