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Mechanisms of Enhanced Resistance of *Mycobacterium bovis* BCG-Treated Mice to Ectromelia Virus Infection

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The mechanism of enhanced resistance of *Mycobacterium bovis* BCG-treated mice to ectromelia virus infection was investigated by determining the effect of splenectomy, antithymocyte serum, and antimacrophage serum on resistance. It was greatly reduced by these treatments, not only in normal mice, but also in mice treated with live or heat-inactivated BCG. Production of circulating interferon by ectromelia virus and Newcastle disease virus was augmented in BCG-treated mice and was markedly depressed by splenectomy and antithymocyte and antimacrophage serum treatments in both BCG-treated and normal mice. Carbon clearance activity was activated in BCG-treated mice, but splenectomy did not influence phagocytic activity. These results suggest that augmented interferon production in the spleens of BCG-treated mice plays a major role in enhanced resistance. Other possible mechanisms are discussed.

Many reports have shown that administration of various agents affecting the reticuloendothelial system (RES) enhances the resistance of animals to viral infections (6, 10, 22, 26, 33). However, the mechanism of increased host resistance is not understood. Our previous experiments (33) have shown that *Mycobacterium bovis* BCG-treated mice acquire increased resistance to ectromelia virus infection (9) and that enhanced interferon production in peritoneal exudate cells and spleen cells may bring about the augmented resistance of BCG-treated mice. The role of interferon in the defense mechanism of the host to natural virus infection has not been clarified, although administration of exogenous interferon or interferon inducers to animals may markedly increase resistance to viral infection (19). Recently, Gresser and colleagues (11-13, 27, 35) and Iwasaki and Nozima (17) demonstrated that the early production of interferon in the response of mice to several viruses was an important factor in host resistance. Numerous studies have discussed the effect of splenectomy on interferon production in several animals in response to various inducers and have reported reduced interferon production in splenectomized animals in response to inoculated virus (7, 23, 32). On the other hand, recent reports have also shown the important role of cellular mechanisms involving immune thymocytes and macrophages in host resistance to various viral infections (1, 8, 24, 34, 37) and that antithymocyte and antimacrophage sera reduce the resistance of mice to virus infection (14, 15, 38).

The purpose of this study was to determine possible mechanisms of increased resistance of BCG-treated mice to viral infection by examining the effects of splenectomy, antithymocyte serum, and antimacrophage serum on such resistance.

MATERIALS AND METHODS

Mice. Eight- to 12-week-old female DDN mice were obtained from the closed colony of the Institute for Experimental Animals, Asahikawa Medical College. One group of mice was inoculated intraperitoneally with 1 mg (wet weight) of BCG (BCG-treated mice), and another group was left uninoculated (untreated mice). Three weeks later, these mice were splenectomized or sham operated and allowed a 1-week recovery period before use in experiments. The splenectomized mice were prepared as follows: under ethyl ether anesthesia, a sagittal skin incision (1 cm) was made on a lumbar area, and then an oblique incision (1 cm) of muscles and peritoneum, parallel to lumbar blood vessels and nerves, was carried out on a splenic area. After ligature of the splenic vessels en masse, the spleen was excised, and the peritoneum, muscles, and skin were sutured as a unit. The sham operation of control mice was done as above except there was no excision of spleen.

Cells. L-929 cells were cultivated in Eagle minimum essential medium supplemented with 10% calf serum. Mouse embryo fibroblast cell cultures were prepared by trypsinization of 16-day-old DDN mouse embryos. The cells were cultivated with Eagle minimum essential medium–10% calf serum.

Viruses. The Ishibashi strain of ectromelia virus was kindly supplied by Y. Ichihashi, Department of Virology, Niigata University School of Medicine, Niigata,

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Japan. The virus was passaged in mouse liver seven times and then in mouse embryo fibroblast cell cultures three times and stored at −80°C. The infectivity titer of the stock ectromelia virus was $10^{6.3}$ 50% lethal doses (LD_{50}) per ml in mice. Purified ectromelia virus used for interferon induction was prepared as follows. The stock ectromelia virus was concentrated by ultracentrifugation on a 50% sucrose cushion and by ultrafiltration (immersible molecular separator; Millipore Corp., Bedford, Mass.) at 4°C. The concentrated ectromelia virus was overlaid on a 15 to 50% (wt/vol) potassium-sodium tartrate density gradient in 0.1 M NaCl–0.001 M EDTA–0.01 M Tris-hydrochloride buffer, pH 7.4, containing 0.1% bovine serum albumin and centrifuged at 52,000 × *g* for 1 h in a Hitachi preparative ultracentrifuge with an RPS-27-2 rotor. Ectromelia virus fractions were collected, dialyzed against 0.01 M phosphate-buffered saline solution (PBS; pH 7.4), and stored at −80°C. The infectivity titer of the purified ectromelia virus was $10^{8.5}$ LD_{50} per ml. The Miyadera strain of Newcastle disease virus was grown in embryonated eggs, and the infectious allantoic fluid was used as an interferon inducer. The infectivity was $10^{9.3}$ PFU/ml. The New Jersey strain of vesicular stomatitis virus was prepared as described in a previous report (2).

Bacteria. BCG was kindly supplied by J. Arima, Institute of Immunological Science, Hokkaido University, Sapporo, Japan, and was grown in Dubos liquid medium (Eiken Chemical Co., Tokyo, Japan) for 4 weeks. The cultures were centrifuged at 3,000 rpm for 15 min. The cells were resuspended in the same medium to 20 mg (wet weight)/ml and stored at −80°C. Heat-inactivated BCG (H-BCG) was prepared by heating at 100°C for 30 min.

Titration of serum interferon. Serial blood samples of 50 μl, used as interferon samples, were harvested from the retroorbital plexus with a micropipette. The samples were mixed with 200 μl of minimum essential medium containing 5 U of heparin per ml and then were centrifuged at 1,500 rpm for 15 min. The supernatant fluids were mixed with an equal volume of anti-ectromelia virus rabbit serum (128 neutralizing units per ml) or anti-Newcastle disease virus rabbit serum (128 hemagglutination inhibiting units per ml) and incubated at 37°C for 30 min and then at 4°C overnight to inactivate viruses in the interferon samples, instead of pH 2 treatment and ultracentrifugation, since the samples were too small to treat by these methods. In preliminary experiments, all interfering activity and interferon-inducing activity of ectromelia and Newcastle disease viruses in the interferon samples were eliminated by treatment with anti-ectromelia and anti-Newcastle disease virus sera, in agreement with an earlier report (12). Interferon was assayed by the micromethod of 50% cytopathic effect reduction in vesicular stomatitis virus-infected L-929 cell cultures as follows: L-929 cell monolayers in Falcon plastic plates (96-well plates) were incubated with 100 μl of serially diluted interferon samples for 18 h at 37°C and then challenged with vesicular stomatitis virus at $10^{1.5}$ 50% tissue culture infectious doses per 100 μl. The interferon titers were expressed as the reciprocal of the maximum dilution showing 50% reduction of cytopathic effect and were calibrated against the mouse reference standard interferon (reagent no. S-002-904-511) kindly supplied by the Research Resources Branch, National Institute of Allergy and Infectious Diseases, Bethesda, Md.

Carbon clearance test. Carbon clearance activity of the RES of mice was measured according to the method described by Stuart (31). Mice were injected intravenously with a colloidal carbon suspension ('Pelikan' special ink C11/1431a, Günther Wagner, Hannover, W. Germany; kindly supplied by H. Komatsu, The Kitasato Institute, Tokyo, Japan) in PBS into the tail vein at a rate of 0.16 mg/0.01 ml per g of mouse body weight. Serial blood samples of 25 μl were obtained from the retroorbital venous plexus with a micropipette at successive time intervals of 1, 2, 3, 6, 9, 12, 15, and 20 min. The samples were mixed with 2 ml of 0.8% (wt/vol) NH_4Cl in 15 mM Tris-hydrochloride buffer (pH 7.4) for hemolysis to occur. The amount of carbon retained in the blood sample was measured by the extinction at 610 nm in a Shimadzu spectrophotometer.

Antisera. Antithymocyte serum was prepared as follows: the thymocytes were collected by straining a minced thymus of a 4- to 6-week-old DDN mouse through stainless-steel mesh. These cells were washed three times with 0.01 M PBS (pH 7.4) and suspended in PBS at a concentration of 2 × 10^6 cells per ml. Rabbits were inoculated intravenously with 1 ml of the cell suspension four times at 1-week intervals and sacrificed 7 days after the last injection, and their blood was collected. The serum (10 ml) was absorbed twice with 1 ml of packed DDN mouse erythrocytes to remove hemolytic activity and inactivated at 56°C for 30 min. The antithymocyte serum diminished peripheral blood leukocytes by 46% within 4 h after a single intraperitoneal inoculation of mice with 1 ml of the serum. The activity of complement-dependent cytotoxicity of antithymocyte serum was 2,560 U/0.2 ml.

Antimacrophage serum was prepared in the same way as antithymocyte serum, except that the macrophages used for immunization were collected as follows: peritoneal cavities of normal mice were washed with PBS, and the peritoneal exudate cells were cultured with Eagle minimum essential medium–10% calf serum for 4 days at 37°C in flat-bottom bottles. The nonadherent cells were washed out; the adherent cells, collected by scraping with a rubber policeman, were suspended in PBS at a concentration of 2 × 10^6 cells per ml and used for immunization. The antimacrophage serum (10 ml) was absorbed twice with 10^8 cells of the nonadherent peritoneal cells to remove antibodies against the leukocytes and lymphocytes. The activity of complement-dependent cytotoxicity of antimacrophage serum was 640 U/0.2 ml. Antithymocyte and antimacrophage sera had no cross-cytotoxicity to macrophages and thymocytes, respectively.

RESULTS

Effect of splenectomy on ectromelia virus infection. One group of BCG-treated and untreated mice was splenectomized, and another group of mice was sham operated. After a 1-week recovery period, these mice were inoculated intraperitoneally with 0.2 ml of serial 10-fold-diluted ectromelia virus, and subsequent deaths were recorded for 3 weeks. The acquired resistance of BCG-treated mice to ectromelia virus infection

was greatly reduced after splenectomy (Table 1). In the case of untreated mice, no difference in resistance was observed between splenectomized and sham operated mice, because the titer of the stock ectromelia virus was the limiting factor. Therefore, we examined the survival patterns of splenectomized mice and sham-operated mice inoculated with ectromelia virus. BCG-treated and untreated mice were inoculated intraperitoneally with 10^2 LD_{50} of ectromelia virus. The results presented in Fig. 1 show that the time of 50% death of the splenectomized mice was earlier than that of the sham-operated group in both BCG-treated and untreated mice.

To determine whether this BCG effect was maintained for a long period, mice were inoculated with 10^2 LD_{50} of ectromelia virus at 1 and 3 months after BCG inoculation. The results presented in Fig. 2 show that mice have a high resistance to ectromelia virus infection 1 month after BCG inoculation, but they show a decreased resistance 3 months after BCG inoculation.

To obtain information on the mechanism of BCG action and on the effect of splenectomy on the outcome of ectromelia virus infection, H-BCG-treated mice and H-BCG-treated and splenectomized mice were inoculated intraperitoneally with 10^2 LD_{50} of ectromelia virus. H-BCG-treated mice had high resistance to ectromelia virus infection, but splenectomized mice showed markedly diminished resistance (Fig. 3). These results suggest that some component(s) of BCG provides augmented resistance, but that growth of BCG in mice is not required.

Effect of splenectomy on interferon production. An experiment was performed to determine the effect of splenectomy on interferon production. In previous experiments (33), interferon production in peritoneal exudate cells of BCG-treated mice in response to ectromelia virus infection was significantly higher than in normal mice, but interferon production in other organs, including spleens, of BCG-treated mice was reduced compared with that in normal mice. These results might reflect a suppressed growth and low dissemination of ectromelia virus from peritoneal cells to other organs. Thus, in this experiment, splenectomized groups and sham-operated groups of BCG-treated and untreated mice were inoculated intravenously with 0.2 ml of purified ectromelia virus (10^7 LD_{50} per 0.2 ml) as an interferon inducer. At successive time intervals thereafter, interferon was measured in the blood samples harvested from the retroorbital venous plexus of each mouse. Interferon production by ectromelia virus was remarkably reduced in the splenectomized mice, but in the BCG-treated and sham-operated mice it was enhanced (Fig. 4). These results suggest that the spleen cells play an important role in interferon production in mice and in resistance to ectromelia virus infection.

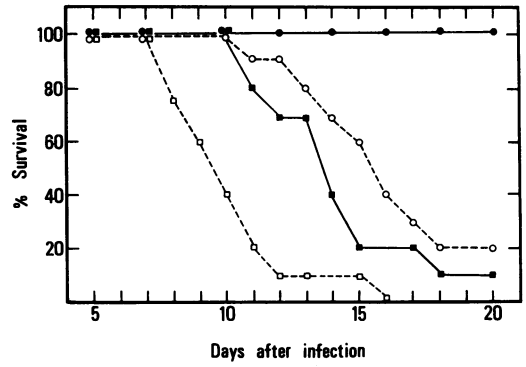

FIG. 1. Effect of splenectomy on mortality after ectromelia virus infection. BCG-treated mice and untreated mice were sham operated or splenectomized. After a 1-week recovery period, these mice were inoculated intraperitoneally with 10^2 LD_{50} of ectromelia virus. Symbols: ●, sham-operated group of BCG-treated mice; ■, splenectomized group of BCG-treated mice; ○, sham-operated group of untreated mice; □, splenectomized group of untreated mice.

TABLE 1. Effect of splenectomy on ectromelia virus infection

Virus dilution[a]	Mortality (no. of mice that died/no. inoculated)			
	Sham-operated mice		Splenectomized mice	
	Untreated	BCG treated	Untreated	BCG treated
10^{-2}	5/5	5/5	ND[b]	ND
10^{-3}	5/5	2/5	5/5	5/5
10^{-4}	5/5	0/5	5/5	5/5
10^{-5}	4/5	0/5	5/5	4/5
10^{-6}	3/5	0/5	5/5	0/5
10^{-7}	0/5	ND	0/5	0/5
Log_{10} LD_{50}	−5.9	−2.9	−6.5	−5.3

[a] A 0.2-ml amount of diluted ectromelia virus was inoculated intraperitoneally.
[b] ND, Not done.

Effect of splenectomy on RES function in BCG-treated and untreated mice. The above results do not eliminate the possibility that the enhanced function of the entire RES of BCG-treated mice also contributes to the increased resistance. Therefore, splenectomized groups and sham-operated groups of BCG-treated and untreated mice were examined for carbon clearance activity as described in Materials and Methods. Figure 5 shows that carbon clearance activity was greatly enhanced in BCG-treated mice and that the activity was unaffected by splenectomy not only in untreated mice, in agreement with an

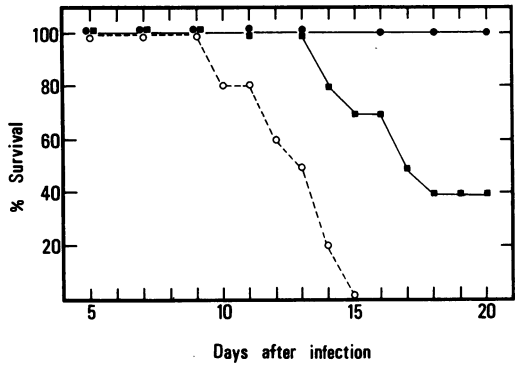

FIG. 2. Duration of enhanced resistance in BCG-treated mice. At 1 or 3 months after BCG treatment, mice were sham operated or splenectomized. After a 1-week recovery period, these mice were inoculated intraperitoneally with 10^2 LD_{50} of ectromelia virus. Symbols: ●, sham-operated group of BCG-treated mice, 1 month after treatment; ■, sham-operated group of BCG-treated mice, 3 months after treatment; ○, splenectomized group of BCG-treated mice, 1 month after treatment.

earlier report (25), but also in BCG-treated mice. The increased resistance of BCG-treated mice to ectromelia virus infection correlates with the enhanced interferon-producing ability in the spleens of these mice, but does not correlate with the phagocytic function of the entire RES.

Effect of antithymocyte serum and antimacrophage serum on ectromelia virus infection. The above results suggest the possibility that the enhanced resistance of BCG- or H-BCG-treated mice to ectromelia virus infection was caused by accentuated interferon production by thymus-derived cells and macrophages. If so, then it might be possible to demonstrate experimentally that antithymocyte and antimacrophage sera will reduce the resistance of mice to ectromelia virus infection, as shown by other virus infections in earlier reports (14, 15, 38). The results presented in Fig. 6 show that enhanced resistance of H-BCG-treated mice was greatly re-

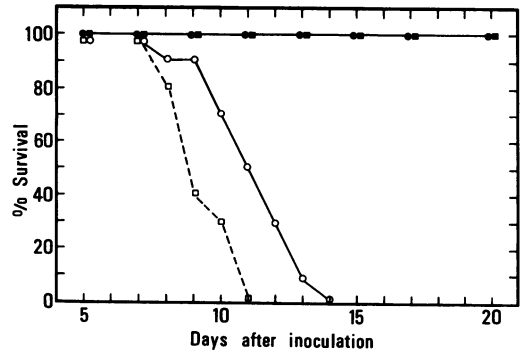

FIG. 3. Resistance of H-BCG-treated mice to ectromelia virus infection and effect of splenectomy on resistance. Symbols: ●, sham-operated group of BCG-treated mice; ■, sham-operated group H-BCG-treated mice; ○, splenectomized group of BCG-treated mice; □, splenectomized group of H-BCG-treated mice.

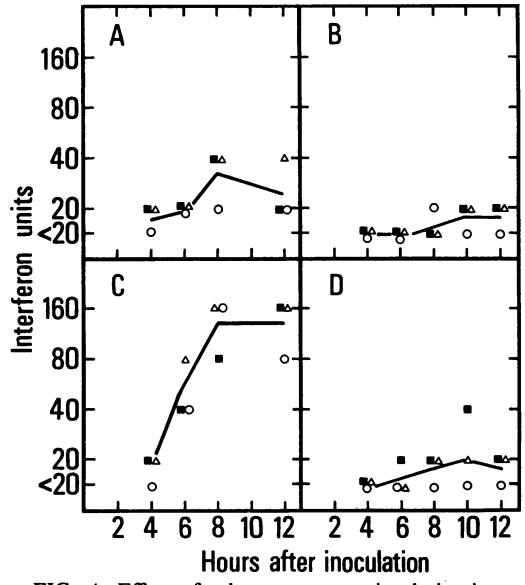

FIG. 4. Effect of splenectomy on circulating interferon production by ectromelia virus in BCG-treated and untreated mice. Untreated and sham-operated mice (A), untreated and splenectomized mice (B), BCG-treated and sham-operated mice (C), and BCG-treated and splenectomized mice (D) were inoculated intravenously with 10^7 LD_{50} of ectromelia virus. Symbols show the interferon units of serial serum samples obtained from the retroorbital plexus of each mouse, and lines show the average interferon units at each period.

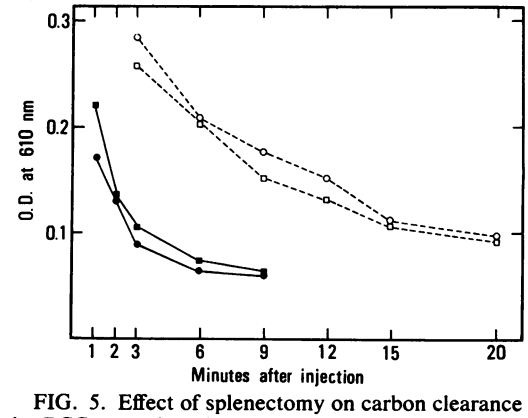

FIG. 5. Effect of splenectomy on carbon clearance in BCG-treated and untreated mice. Symbols: ●, sham-operated group of BCG-treated mice; ■, splenectomized group of BCG-treated mice; ○, sham-operated group of untreated mice; □, splenectomized group of untreated mice. Each group consisted of three mice, and average values were plotted. O.D., Optical density.

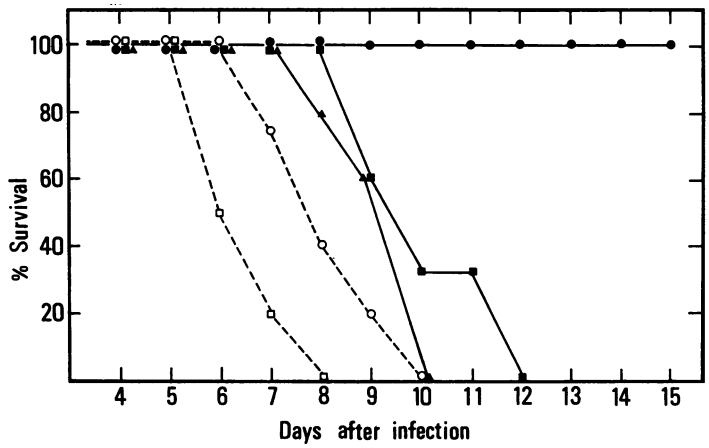

FIG. 6. Effect of antithymocyte and antimacrophage sera on mortality after ectromelia virus infection. BCG-treated and untreated mice were inoculated intraperitoneally with 1 ml of antithymocyte or antimacrophage serum three times at 1-day intervals. Then, the mice were inoculated intraperitoneally with 10^2 LD_{50} of ectromelia virus. Symbols: ●, BCG-treated mice not inoculated with antiserum; ■, BCG-treated mice inoculated with antithymocyte serum; ▲, BCG-treated mice inoculated with antimacrophage serum; ○, untreated mice not inoculated with antiserum; □, untreated mice inoculated with antithymocyte serum.

duced by treatment with anti-thymocyte or anti-macrophage serum; i.e., it was reduced to the resistance levels of the untreated mice.

Effects of splenectomy, antithymocyte serum, and antimacrophage serum on interferon production in BCG-treated and untreated mice. It has been shown that splenectomized mice produce a reduced amount of circulating interferon in response to ectromelia virus (Fig. 4). We next examined circulating interferon production in mice treated by splenectomy and with antithymocyte (4, 5) and antimacrophage sera. Because of the low interferon inducibility of ectromelia virus, circulating interferon levels in these treated mice was very low, and no significant difference in the effect of single, double, or triple treatment on circulating interferon levels in both BCG-treated and untreated mice was detected (data not shown). Therefore, the effect of splenectomy, antithymocyte serum, or antimacrophage serum, or all three, on the circulating interferon levels in mice in response to Newcastle disease virus, which has a high interferon-inducing ability, was examined. The circulating interferon levels of both untreated (Fig. 7A and B) and H-BCG-treated (Fig. 7C and D) mice were reduced by splenectomy (Fig. 7B and D) or treatment with antimacrophage serum and remarkably reduced by the triple treatment with antithymocyte serum, antimacrophage serum, and splenectomy to about 1 to 2% of control values. When the mice had been treated with H-BCG and showed enhanced circulating interferon production, treatment by splenectomy and with antithymocyte or antimacrophage serum reduced the circulating interferon levels in H-BCG-treated mice to the same levels as in untreated mice. The same pattern was also obtained in BCG-treated and untreated mice (data not shown).

DISCUSSION

The experiments reported in this paper have shown clearly that the augmented resistance of BCG-treated mice to viral infection is reduced by splenectomy and by treatment with antithymocyte and antimacrophage sera and also that the augmented interferon production in BCG-treated mice is diminished by such treatments. These results suggest that, in this animal-virus system, the main interferon-producing cells are thymus-derived cells and macrophages of the whole body, especially of spleens, and that these cells are elicited by BCG or H-BCG and participate in the enhanced interferon production. We conclude that this interferon production is probably related to the enhanced resistance in either BCG- or H-BCG-treated mice.

An earlier report (33) showed that the final target organ of intraperitoneally inoculated ectromelia virus was the liver. Carbon clearance activity of mice, in which 90% of intravenously inoculated carbon is cleared by the liver, was enhanced in BCG-treated mice, but was not reduced by splenectomy. These observations indicate that the enhanced phagocytic activity of the entire RES, especially of the liver, does not contribute to the increased resistance of BCG-treated mice to ectromelia virus and that the augmented interferon production of thymus-derived cells and macrophages in spleens of BCG-

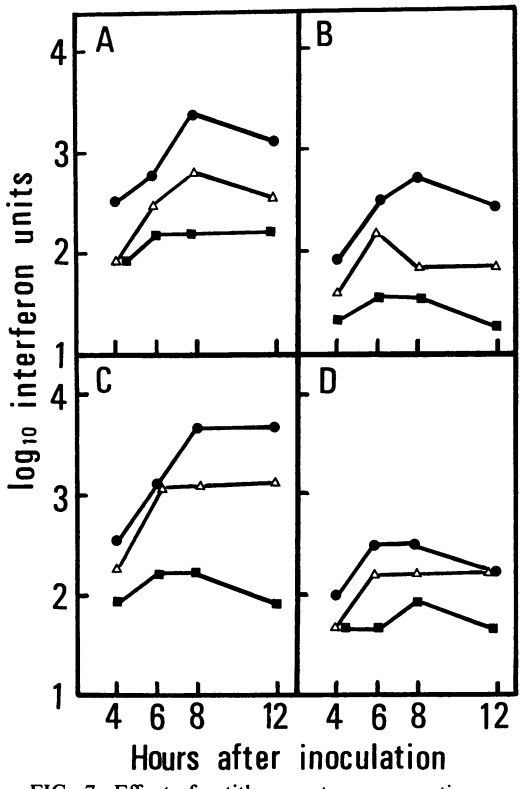

FIG. 7. Effect of antithymocyte serum, antimacrophage serum, and splenectomy on interferon production in H-BCG-treated and untreated mice. Sham-operated and untreated mice (A), splenectomized and untreated mice (B), sham-operated and H-BCG-treated mice (C), and splenectomized and H-BCG-treated mice (D) were inoculated intraperitoneally with 1 ml of antithymocyte or antimacrophage serum or both three times at 1-day intervals. Then the mice were inoculated intravenously with 10^7 PFU of Newcastle disease virus per 0.2 ml. Serial blood samples were harvested from the retroorbital plexus of each mouse of each group of 3 mice. The interferon titer was assayed and the average unit was plotted. Symbols: ●, mice not treated with antiserum; △, mice treated with antimacrophage serum; ■, mice treated with both antisera.

treated mice might play a major role in resistance. In contrast with our results, Larson et al. (20) and Lodmell and Ewalt (21) reported that the interferon was not detected in sera of *Mycobacterium tuberculosis*- or BCG-treated mice after virus inoculation, and they suggested that the importance of systemic interferon in enhanced resistance of these mice to virus infection was doubtful. These discrepancies may reflect the doses of virus inoculated or the different target organs for the virus used. As shown in our previous report (33), the production of circulating interferon in BCG-treated mice inoculated intraperitoneally with 10^3 LD_{50} of ectromelia virus was lower than in untreated mice because of suppressed growth and low dissemination of ectromelia virus in the BCG-treated mice. In the present study, BCG-treated mice were inoculated with a high titer of ectromelia or Newcastle disease virus as an interferon inducer to amplify the interferon-producing ability of these mice, which showed an enhanced ability of interferon production (Fig. 4 and 7). These results suggest that the growth of ectromelia virus in BCG-treated mice may be depressed in the primary target organ by the enhancement of early interferon production, as shown in an earlier report (17). In this case, dissemination of virus to other organs from the primary target organ may be suppressed, and the interferon levels in mice are low.

Recently, Rodda and White (28) reported a role for cytotoxic macrophages in nonspecific defense mechanisms of the host to virus infection, and a number of studies have demonstrated the importance of macrophage action in the primary defense against virus infections (3, 16, 24, 29, 30, 39). On the other hand, many reports have shown the important role of cellular mechanisms involving immune thymocyte and natural killer cells in host resistance to virus infections (8, 15, 18, 34, 36, 37). The relationship between the cytotoxic activity of macrophages, immune thymocytes, and natural killer cells and the enhanced interferon production in spleens of BCG-treated mice is now being investigated in connection with the increased resistance of these mice.

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