Role of Interferon in the Augmented Resistance of Trehalose-6,6′-dimycolate-treated Mice to Influenza Virus Infection

By MASANOBU AZUMA,* TATSUO SUZUTANI, KATSUHIKO SAZAKI, ITSUROU YOSHIDA, TAKASHI SAKUMA AND TAKESHI YOSHIDA
Department of Microbiology, Asahikawa Medical College, Asahikawa 078, Japan

(Accepted 20 November 1986)

SUMMARY
Mice inoculated intravenously with 10 to 100 μg trehalose-6,6′-dimycolate in an oil-in-water emulsion (TDM emulsion) acquired high resistance to intranasal infection by influenza virus at 7 to 14 days, but not at 1 day, after treatment. Mice inoculated with an oil-in-water emulsion without TDM (control emulsion) did not resist infection. The activity of the reticuloendothelial system of mice inoculated with TDM emulsion or control emulsion was greatly stimulated 1 day and 14 days after treatment. Interferon production in response to influenza virus was augmented in lung and serum of TDM emulsion-treated mice. The augmented interferon production was greatly diminished in the TDM emulsion-treated mice by treatment with anti-Thy-1.2 monoclonal antibody. Production of haemagglutination-inhibiting antibody in the TDM emulsion-treated or control emulsion-treated mice was higher than that in untreated mice, although no difference was observed between the TDM emulsion-treated and control emulsion-treated mice. On the other hand, TDM emulsion treatment of mice did not influence the appearance of antibody-producing cells, nor the activity of natural killer cells in the mice. The enhanced resistance of mice was diminished by inoculating anti-interferon-α/β serum before influenza virus infection. No detectable interferon activity was observed in lung and blood of mice inoculated with anti-interferon-α/β serum prior to influenza virus infection. These results suggest that the augmented early interferon production in T-lymphocytes of TDM emulsion-treated mice in response to influenza virus may play an important role in the enhanced resistance.

INTRODUCTION
In our previous reports, we have shown that mice inoculated with heat-inactivated BCG acquired a high non-specific resistance to virus infection, and that this acquired resistance was due mainly to enhanced interferon production in the BCG-inoculated mice (Suenaga et al., 1978; Sakuma et al., 1983). We have also shown that N-acetyl-muramyl-L-alanyl-D-isoglutamine (MDP), which is the minimal active portion of the peptidoglycan of the bacterial cell wall for adjuvant activity (Ellouz et al., 1974; Adam et al., 1976; Kusumoto et al., 1976; Tanaka et al., 1979), has no enhancing activity on resistance to virus infection (Sakuma et al., 1984), though MDP has been reported to potentiate non-specific resistance to bacterial and mycotic infection (Chedid et al., 1977; Cummings et al., 1980; Yamamoto et al., 1980; Matsumoto et al., 1981).

Trehalose-6,6′-dimycolate (cord factor; TDM) is a glycolipid component of the cell wall of Mycobacterium, Corynebacterium and Nocardia, and has haptenic activity (Ozawa et al., 1957), granulomagenic activity (Bekierkunst, 1968; Bekierkunst et al., 1969; Barkdale & Kim, 1977; Yamamoto et al., 1980), adjuvant activity (Bekierkunst et al., 1971; Granger et al., 1976) and anti-tumour activity (Goren & Brennan, 1979). Bekierkunst (1968) and Yarkoni & Bekierkunst (1976) have reported that TDM enhanced non-specific resistance to bacterial infections, although the mechanism remains to be defined. The purpose of the present study was to determine whether TDM enhanced non-specific resistance of mice to influenza virus infection, and to analyse the mechanism.
METHODS

Mice. Eight- to 12-week-old female ddN mice were supplied from the closed colony of the Animal Laboratory for Medical Research of our college.

Viruses. The A/PR/8/34/(H1N1) strain of influenza virus, modified by adaptation to mouse lung, was kindly supplied by Dr N. Ishida, Department of Bacteriology, Tohoku University School of Medicine, Sendai, Japan. The stock virus was passaged three times in mouse lung by intranasal infection and then once in 11-day-old embryonated eggs in our laboratory, and stored in small aliquots at -80 °C. The infectivity of the stock virus in mice was 10^4.5 50% lethal doses (LD_{50}) per ml.

TDM and control adjuvant. TDM, prepared from Mycobacterium bovis, strain ANS, was purchased from Chaoy Chimie Reactifs, Paris, France and the oil-in-water emulsion of TDM was prepared by the method of Yamamoto et al. (1980) with modifications as follows: 10 mg TDM was dissolved in 100 ml ethyl ether in a tightly closed bottle, and stored at 4 °C as stock TDM solution. For each experiment, about 1-5 ml of the stock solution was transferred into a glass vessel of a 5 ml Teflon homogenizer (Wheaton Scientific, Millville, N.J., U.S.A.) and the ethyl ether was evaporated at room temperature. The TDM residue (150 µg from a 1-5 ml stock solution) was ground with 30 µl Freund’s incomplete adjuvant. Then, 3 ml phosphate-buffered saline solution containing 0-2% Tween 80 (Tween 80-PBS pH 7-4) was added to the above mixture, and ground again to make an oil-in-water emulsion. For the control experiments, the oil-in-water emulsion was prepared with Freund’s incomplete adjuvant and Tween 80-PBS without TDM. These emulsions are referred to as TDM emulsion and control emulsion, respectively. Mice were intravenously inoculated with 0-2 ml of these emulsions (thus, for 1-5 ml of initial sample, 10 µg TDM in 0-2 ml emulsion was inoculated; these will be referred to as TDM-10 emulsion-inoculated mice). After designated periods these mice were used for experiments. The minimum LD_{50} of TDM in mice was 6.4 mg/kg.

Antisera. Anti-Thy-1.2 monoclonal antibody (mouse ascites fluid; ATMA) was generously supplied by Dr H. Katagiri and Dr H. Yakura, the 2nd Department of Pathology, Asahikawa Medical College, Asahikawa, Japan. The activity of complement-dependent cytotoxicity of ATMA was 2.5 x 10^5 units/0.25 ml.

Anti-interferon-α/β serum, prepared by immunizing sheep with 12 weekly injections of 1 x 10^7 units/mg protein; containing mouse interferon-α and -β by removing greater than 95% of the antibodies to L-929 cell components present in the interferon preparation used for immunization by immunoabsorption techniques utilizing antigens bound to Sepharose-4B, was generously supplied by Dr Y. Kawade, Institute for Virus Research, Kyoto University, Kyoto, Japan. The activity of this serum was 10^6 neutralizing units/ml for both interferon-α and -β (Kawade, 1980).

Treatment of mice with antisera. TDM emulsion-treated and control emulsion-treated mice or untreated mice were intraperitoneally inoculated with 10200 units/ml ATMA 1 and 2 days before virus infection and Tween 80-PBS without TDM. These emulsions are referred to as TDM emulsion and control emulsion, respectively. Mice were intravenously inoculated with 0-2 ml of these emulsions (thus, for 1-5 ml of initial sample, 10 µg TDM in 0-2 ml emulsion was inoculated; these will be referred to as TDM-10 emulsion-inoculated mice).

Titration of TDM and control adjuvant. TDM emulsion-treated and control emulsion-treated mice or untreated mice were intraperitoneally inoculated with 10200 units/ml ATMA 1 and 2 days before virus infection, and with 10^5 units/ml anti-interferon-α/β serum 4 h prior to infection.

Titration of interferon in serum and in lung. Serial blood and lung samples were harvested at successive time intervals from mice inoculated intranasally with 10^4.5 LD_{50} in 25 µl (about 10^4.5 50% egg infectious doses) of influenza virus. The lung was ground by pestle and mortar, suspended in Eagle’s MEM at a concentration of 10% (w/v) and centrifuged at 3000 r.p.m. for 20 min. An aliquot of each serum or lung extract was mixed with an equal volume of anti-influenza virus rabbit serum [128 haemagglutination-inhibiting (HI) units/ml] and incubated at 37 °C for 30 min to inactivate viruses in the interferon samples (Gresser et al., 1976a; Sakuma et al., 1983). Interferon activity was assayed by 50% c.p.e. reduction in vesicular stomatitis virus-infected L-929 cell cultures as described previously (Sakuma et al., 1983). The mouse international reference research standard (reagent number S-002-905-511, kindly supplied by the Research Resources Branch, NIAID, Bethesda, Md., U.S.A.) was designated to have an activity of 12 000 units/ml, and experimental titres were standardized by using this standard interferon.

Carbon clearance test. Measurements of the carbon clearance activity of the reticuloendothelial system (RES) of mice were made as described in the previous report (Sakuma et al., 1983).

Titration of HI antibody. The HI antibody to influenza virus was assayed by the micromethod as described by Sever (1962). The antibody activity is expressed as the reciprocal of the maximum dilution of serum showing complete inhibition of haemagglutination.

Plaque technique for detecting antibody-producing cells. The plaque-forming assay was performed with spleen cells of sheep red blood cell (SRBC)-immunized mice by the method of Cunningham & Szenberg (1968).

Assay for natural killer cell activity. The assay was carried out using a modification of the [3H]uridine-release assay method described by Hashimoto & Sudo (1971). Briefly, target cells (YAC-1 cells derived from Moloney virus-induced mouse lymphoma, 2 x 10^5 cells) were labelled with 10 µCi/ml [5-3H]uridine (27 Ci/mmol; New England Nuclear) for 4 h at 37 °C, and suspended in RPMI 1640 medium (Nissui Seiyaku Co., Tokyo, Japan) supplemented with 10% foetal bovine serum (RPMI 1640-FS10) at a concentration of 1 x 10^6 cells/ml. Lymphocytes as effector cells were prepared from mouse spleen cells, and suspended in RPMI 1640-FS10 at a concentration of 1 x 10^6 and 1 x 10^7 cells/ml. Target cells and lymphocytes were mixed (0.5 ml each), and 50 µl 50 mM unlabelled uridine was added to the cell mixture to block reincorporation of released [3H]uridine. The cell
mixture was incubated in a 5% CO₂ incubator at 37 °C for 4 h. After centrifugation, the radioactivity of 0.5 ml of the supernatant fluid was measured. Autologous controls were employed for base-line release values in all experiments. The specific cytotoxicity was calculated by the formula of Hashimoto & Sudo (1971).

RESULTS

Effect of TDM on influenza virus infection

TDM-10 emulsion-inoculated or control emulsion-inoculated mice and uninoculated mice were infected intranasally with 10² LD₅₀ in 25 μl influenza virus, and subsequent deaths were recorded for 2 weeks. The results presented in Fig. 1 show that the mice inoculated with TDM emulsion at 7 days (data not shown) or 14 days before infection acquired high resistance to influenza virus infection, but not the mice inoculated with TDM-10 emulsion 1 day before infection (data not shown) or with control emulsion 14 days before infection. The enhanced resistance was greatly diminished in TDM-10 emulsion-inoculated mice by treatment prior to infection with ATMA. These results suggest that some function of thymus-dependent lymphocytes provides augmented resistance.

Effective doses of TDM were then determined. As shown in Table 1, the 50% minimum effective dose of TDM was 2.5 μg/mouse.

In these experiments, mice were treated with TDM-10 emulsion or control emulsion 14 days before virus infection.

Effect of TDM on function of reticuloendothelial system (RES) in mice

The above results do not preclude the possibility that the enhanced function of the entire RES of TDM emulsion-inoculated mice also contributes to the augmented resistance. Therefore, TDM-10 emulsion-inoculated or control emulsion-inoculated mice were examined for carbon clearance activity. Fig. 2 shows that carbon clearance activity was greatly enhanced in TDM emulsion-inoculated mice, and also in control emulsion-inoculated mice, in which enhanced resistance was not shown. These results indicate that the entire RES function in mice does not correlate with the enhanced resistance to virus infection, as in our earlier report (Sakuma et al., 1983).

Interferon production in lung and serum of TDM emulsion-inoculated and influenza virus-infected mice

In our previous report (Suenaga et al., 1978), the production of circulating interferon in BCG-treated mice inoculated with low doses of ectromelia virus was lower than that in untreated mice because of the suppressed growth and slower initial dissemination of virus. When these BCG-treated mice were inoculated with a high dose of virus as an interferon inducer, their interferon-producing ability was amplified (Sakuma et al., 1983). In the present experiment, TDM emulsion- or control emulsion-treated mice were inoculated intranasally with a high dose (10⁵ LD₅₀ in 25 μl) of influenza virus, and lung and serum were sampled at 12 and 24 h. As shown in Table 2, augmented interferon production in lung and serum in TDM emulsion-inoculated mice was observed, and interferon production was eliminated by treatment of these mice with ATMA. TDM emulsion alone did not induce interferon production (data not shown), indicating that the enhanced production of interferon was not caused by a priming effect of TDM emulsion.

Effect of TDM on production of antibody in mice

Production of HI antibody in the TDM emulsion-treated mice and the control emulsion-treated mice 7 days after influenza virus infection (10² LD₅₀, intranasally) was higher than that in untreated mice. No difference, however, was observed between TDM emulsion-treated mice and control emulsion-treated mice (Table 3), indicating that higher antibody production in TDM emulsion- and control emulsion-treated mice than that in untreated mice might be caused by the adjuvant activity of Freund's incomplete adjuvant, and not by the function of TDM. On the other hand, treatment of mice with TDM emulsion did not influence the appearance of antibody-producing cells when mice were immunized with SRBC (Table 4).
Fig. 1. Effect of pretreatment with TDM emulsion or ATMA on mortality after influenza virus infection. Six mice per group were intravenously inoculated with 10 μg TDM in 0.2 ml oil-in-water emulsion (TDM-10 emulsion) or control emulsion. These mice were intranasally inoculated with 10^2 LD₅₀ in 25 μl influenza virus at 1 or 14 days after TDM treatment. One group of TDM-treated mice were intraperitoneally inoculated with 1 ml ATMA (10200 units/ml) at 1 and 2 days before virus infection. ○, TDM-10 emulsion-treated at 14 days before virus infection; ●, TDM-10 emulsion-treated at 14 days plus ATMA-treated at 1 and 2 days before virus infection; ■, control emulsion-treated at 14 days before virus infection; □, untreated.

Fig. 2. Effect of pretreatment with TDM emulsion or control emulsion on carbon clearance activity in mice (three mice/group) 14 days later. ○, TDM-10 emulsion-treated; ■, control emulsion-treated; □, untreated.

Table 1. Effect of TDM on influenza virus infection

<table>
<thead>
<tr>
<th>Mice pretreated with</th>
<th>Mortality after influenza virus infection †</th>
</tr>
</thead>
<tbody>
<tr>
<td>TDM-100 emulsion</td>
<td>0/4 ‡</td>
</tr>
<tr>
<td>TDM-40 emulsion</td>
<td>0/4</td>
</tr>
<tr>
<td>TDM-10 emulsion</td>
<td>0/4</td>
</tr>
<tr>
<td>TDM-2.5 emulsion</td>
<td>2/4</td>
</tr>
<tr>
<td>TDM-0.6 emulsion</td>
<td>4/4</td>
</tr>
<tr>
<td>Control emulsion</td>
<td>4/4</td>
</tr>
<tr>
<td>None</td>
<td>4/4</td>
</tr>
</tbody>
</table>

* Mice (four per group) were inoculated intravenously with 0.6 to 100 μg TDM in 0.2 ml oil-in-water emulsion (referred to as TDM-0.6 emulsion or TDM-100 emulsion) or control emulsion.
† Mice were intranasally inoculated with 10^2 LD₅₀ in 25 μl influenza virus 14 days after TDM treatment.
‡ Number of dead mice at 14 days after virus inoculation/number of mice inoculated.

Table 2. Effect of TDM on interferon production in lung and serum of influenza virus-infected mice

<table>
<thead>
<tr>
<th>Interferon units after inoculation of influenza virus (h) *</th>
<th>Lung</th>
<th>Serum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mice pretreated with</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control emulsion</td>
<td>12</td>
<td>24</td>
</tr>
<tr>
<td>TDM-10 emulsion</td>
<td>48</td>
<td>192</td>
</tr>
<tr>
<td>TDM-10 emulsion + ATMA†</td>
<td>&lt;12</td>
<td>48</td>
</tr>
</tbody>
</table>

* Two mice per group were intranasally inoculated with 10^5.5 LD₅₀ of influenza virus at 14 days after treatment with TDM or control emulsion.
† Mice pretreated with TDM-10 emulsion and control emulsion were inoculated with ATMA at 1 and 2 days before influenza virus infection.
Table 3. Effect of TDM on production of HI antibody in influenza virus-infected mice

<table>
<thead>
<tr>
<th>Mice pretreated with</th>
<th>HI units in serum after infection (days)*</th>
<th>3</th>
<th>5</th>
<th>7</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>&lt;64</td>
<td>&lt;64</td>
<td>128</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>&lt;64</td>
<td>&lt;64</td>
<td>128</td>
<td></td>
</tr>
<tr>
<td>Control emulsion</td>
<td>&lt;64</td>
<td>&lt;64</td>
<td>1024</td>
<td></td>
</tr>
<tr>
<td>Control emulsion</td>
<td>&lt;64</td>
<td>256</td>
<td>1024</td>
<td></td>
</tr>
<tr>
<td>TDM-10 emulsion</td>
<td>&lt;64</td>
<td>&lt;64</td>
<td>512</td>
<td></td>
</tr>
<tr>
<td>TDM-10 emulsion</td>
<td>&lt;64</td>
<td>&lt;64</td>
<td>1024</td>
<td></td>
</tr>
</tbody>
</table>

* Two mice per group were intranasally inoculated with $10^2 \text{LD}_{50}$ of influenza virus at 14 days after treatment with TDM-10 emulsion or control emulsion.

Table 4. Effect of TDM on appearance of antibody-producing cells

<table>
<thead>
<tr>
<th>Mice pretreated with</th>
<th>Immunized with*</th>
<th>Antibody-producing cells/spleen†</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>None</td>
<td>158 ± 45</td>
</tr>
<tr>
<td>None</td>
<td>SRBC</td>
<td>3529 ± 1144§</td>
</tr>
<tr>
<td>Control emulsion</td>
<td>None</td>
<td>109 ± 56</td>
</tr>
<tr>
<td>Control emulsion</td>
<td>SRBC</td>
<td>3768 ± 945§</td>
</tr>
<tr>
<td>TDM-10 emulsion</td>
<td>None</td>
<td>64 ± 19</td>
</tr>
<tr>
<td>TDM-10 emulsion</td>
<td>SRBC</td>
<td>4989 ± 439†</td>
</tr>
</tbody>
</table>

* Mice were immunized intravenously with 0.2 ml 1% SRBC suspension at 14 days after treatment with TDM emulsion or control emulsion.
† Antibody-producing cells were measured 4 days after immunization. Mean ± standard deviation of four mice is shown.
‡ These values were statistically not significantly different.

Table 5. Natural killer cell activity in TDM-treated mice and control mice

<table>
<thead>
<tr>
<th>Mice pretreated with</th>
<th>Induction with influenza virus or poly(rI)·poly(rC)*</th>
<th>Interferon units in blood</th>
<th>Specific activity of natural killer cells (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>None</td>
<td>ND†</td>
<td>9.1 ± 2.4</td>
</tr>
<tr>
<td>None</td>
<td>Influenza virus</td>
<td>186 ± 122‡</td>
<td>19.3 ± 5.0</td>
</tr>
<tr>
<td>None</td>
<td>Poly(rI)·poly(rC)</td>
<td>8533 ± 2956§</td>
<td>32.2 ± 5.5</td>
</tr>
<tr>
<td>Control emulsion</td>
<td>None</td>
<td>ND</td>
<td>8.9 ± 3.3</td>
</tr>
<tr>
<td>Control emulsion</td>
<td>Influenza virus</td>
<td>266 ± 92‡</td>
<td>21.7 ± 6.8</td>
</tr>
<tr>
<td>TDM-1 emulsion</td>
<td>None</td>
<td>ND</td>
<td>9.9 ± 3.6</td>
</tr>
<tr>
<td>TDM-1 emulsion</td>
<td>Influenza virus</td>
<td>186 ± 122‡</td>
<td>20.8 ± 1.6</td>
</tr>
<tr>
<td>TDM-10 emulsion</td>
<td>None</td>
<td>ND</td>
<td>8.7 ± 1.5</td>
</tr>
<tr>
<td>TDM-10 emulsion</td>
<td>Influenza virus</td>
<td>586 ± 92‡</td>
<td>20.1 ± 4.0</td>
</tr>
</tbody>
</table>

* Mice were intranasally inoculated with $10^{12} \text{LD}_{50}$ of influenza virus, or intravenously with 100 µg poly(rI)·poly(rC) at 14 days after treatment with control emulsion or TDM emulsion.
† ND, Not done.
‡ Mean of three mice ± standard deviation at 24 h post-induction.
§ Mean of three mice ± standard deviation at 2 h post-induction.
¶ Mean of duplicate assay of three mice ± standard deviation at 24 h post-induction.

Natural killer cell activity in TDM-treated mice

It has been reported that natural killer cells may be an important factor in host resistance to virus infection (Santoli et al., 1978; Bancroft et al., 1981; Shellam et al., 1981; Kirchner et al., 1982; Kohl & Harmon, 1983). We therefore examined whether cytotoxic activity of the natural killer cells was enhanced in mice treated with TDM. Each group of TDM-10 emulsion-, TDM-1 emulsion- and control emulsion-treated mice and untreated mice was divided into two sets (three mice/set). One set of each group was intranasally inoculated with $10^{12} \text{LD}_{50}$ of influenza virus, and the other set was uninoculated. After 24 h, spleen cells and sera were prepared from each mouse. The cytotoxicity of natural killer cells in spleen cells and the activity of circulating interferon were then assayed. As shown in Table 5, natural killer cells were not activated in the
The present results have shown that mice treated with TDM emulsion acquired enhanced resistance to influenza virus infection, and that the augmented resistance may be attributed to the enhanced production of interferon in the early period of influenza virus infection, but not to the whole RES and humoral antibody production in the late period. Tsukui (1977) demonstrated that T-lymphocytes are the main interferon producer in response to influenza virus. Table 2 shows that the enhanced interferon production was greatly diminished by treatment of mice with ATMA, suggesting that T-lymphocytes play a major role in interferon production in response to influenza virus; this is in agreement with the report of Tsukui and our earlier report using ectromelia virus-infected mice (Sakuma et al., 1983). At this time, it is not clear whether TDM emulsion-treated mice. An enhanced cytotoxicity was detected in both TDM emulsion- and control emulsion-treated mice in response to influenza virus infection, indicating an augmentation of natural killer cell activity by interferon produced in response to virus infection. In this experiment, no significant difference in the level of augmented activity of natural killer cells was observed between TDM emulsion-treated mice and control mice. Mice inoculated with poly(rI)-poly(rC) as a control inducer showed a higher activity of natural killer cells and yielded about 20 to 40 times more circulating interferon than that produced in influenza virus-inoculated mice.

**Effect of anti-interferon serum on augmented resistance of TDM emulsion-treated mice**

The above results suggest that augmented early interferon production by T-lymphocytes in response to influenza virus infection may play an important role in the enhanced resistance of TDM emulsion-treated mice. If so, then it might be possible to demonstrate experimentally that anti-interferon serum, introduced exogenously, diminishes interferon production in TDM-treated mice and reduces the augmented resistance to influenza virus infection.

The results presented in Fig. 3 show that the enhanced resistance of TDM emulsion-treated mice was markedly reduced by treatment of these mice with anti-interferon-α/β serum prior to infection. Finally, interferon production in lung and serum of TDM emulsion-treated and anti-interferon-α/β serum-treated mice in response to influenza virus infection was examined. No detectable interferon level was observed in lung and serum of TDM plus anti-interferon serum-treated mice (Table 6).

**DISCUSSION**

The present results have shown that mice treated with TDM emulsion acquired enhanced resistance to influenza virus infection, and that the augmented resistance may be attributed to the enhanced production of interferon in the early period of influenza virus infection, but not to the whole RES and humoral antibody production in the late period. Tsukui (1977) demonstrated that T-lymphocytes are the main interferon producer in response to influenza virus. Table 2 shows that the enhanced interferon production was greatly diminished by treatment of mice with ATMA, suggesting that T-lymphocytes play a major role in interferon production in response to influenza virus; this is in agreement with the report of Tsukui and our earlier report using ectromelia virus-infected mice (Sakuma et al., 1983). At this time, it is not clear whether...
TDM directly activates T-lymphocytes or does so indirectly by means of primary activation of macrophages. Earlier reports (Bekierkunst, 1968; Bekierkunst et al., 1969; Barksdale & Kim, 1977; Yamamoto et al., 1980) have shown that TDM has a granulomagenic activity, and that foci of granuloma consist of one or a few macrophages in the centre and accumulated lymphocytes at the periphery. In the lungs of TDM emulsion-treated mice, granuloma were formed in accord with these reports, and the degree of granuloma formation increased with the dose of inoculated TDM and the level of resistance to influenza virus infection; microscopic observation of lung sections showed that the macrophages phagocytosed oil droplets (unpublished data). These results suggest that the primary target cells of TDM may be macrophages which produce a monokine having chemotactic activity for lymphocytes. The non-specific accumulation of lymphocytes may participate in the augmented resistance of mice and enhanced early interferon production. Gresser and colleagues (1976a, b; Rivière et al., 1977), using anti-interferon serum, also showed an important role of early interferon production in the resistance of mice to virus infection. Our experiments with anti-Thy-1.2 monoclonal antibody and anti-interferon-α/β serum showed that the interferon produced by T-lymphocytes in TDM-treated mice in response to immunologically non-specific influenza virus was interferon-α or -β, or both.

It has been reported that natural killer cells play a role in the defence mechanism against virus infection (Santoli et al., 1978; Bancroft et al., 1981; Shellam et al., 1981; Kirchner et al., 1982; Kohl & Harmon, 1983), and that the cytotoxicity of natural killer cells can be increased by non-specific stimulation with BCG (Wolfe et al., 1976, 1977; Tracey, 1979; Potter & Moore, 1980), Corynebacterium parvum (Oehler et al., 1978; Ojo et al., 1978), and poly(rI).poly(rC) or interferon (Oehler et al., 1978; Oehler & Herberman, 1978; Djeu et al., 1979; Welsh, 1981). The results presented in Table 5 showed that there is no increase of cytotoxic activity of natural killer cells in TDM emulsion-treated mice. An augmented cytotoxicity, however, was observed in TDM emulsion-treated mice and control mice after virus infection, though there was no significant difference between the augmented cytotoxicity of these mice. The increased interferon from T-lymphocytes in response to influenza virus infection acts to stimulate natural killer cells. Recently, it has been reported that natural killer cells may play an important role in virus infection through their own interferon production (Welsh, 1981). The augmented early interferon production in lung in response to influenza virus infection and the stimulated activity of natural killer cells may function to restrict the early virus replication and its spread to organs.

Further investigations are required to determine whether macrophages do respond initially to TDM, and accumulate T-lymphocytes and natural killer cells, and also by what means these effects are achieved.

Table 6. Interferon production in response to influenza virus in TDM-treated and anti-interferon serum-treated mice

<table>
<thead>
<tr>
<th>Interferon units after inoculation of influenza virus (h)*</th>
<th>Lung</th>
<th>Serum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mice pretreated with</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control emulsion</td>
<td>12</td>
<td>24</td>
</tr>
<tr>
<td>TDM-10 emulsion</td>
<td>96</td>
<td>192</td>
</tr>
<tr>
<td>TDM-10 emulsion + anti-IFN†</td>
<td>&lt;6</td>
<td>&lt;6</td>
</tr>
</tbody>
</table>

* Two mice per group were intranasally inoculated with 10⁵ LD₅₀ of influenza virus at 14 days after treatment with TDM emulsion or control emulsion.
† Mice were intraperitoneally inoculated with 1 ml anti-interferon-α/β sheep serum (10⁵ neutralizing units/ml) 4 h before inoculation with influenza virus.
The authors wish to thank Dr Yoshimi Kawade, Institute of Virus Research, Kyoto University, Kyoto, Japan, for generously supplying anti-interferon serum, Dr Hajime Katagiri and Dr Hidetaka Yakura, the 2nd Department of Pathology of our college, for kindly supplying anti-Thy-1.2 monoclonal antibody, and Professor Derek C. Burke, Allelix Inc., Ontario, Canada, for advice and critical reading of the manuscript. The excellent technical assistance of Mrs C. Hatanaka is acknowledged.

REFERENCES


Mechanisms of augmented resistance of TDM-treated mice


(Received 8 July 1986)