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Mechanism of Recovery from Acute Virus Infection. VI. Replication of Lymphocytic Choriomeningitis Virus in and Clearance from the Foot of the Mouse

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SUMMARY

The hind foot was chosen for study of the mechanism by which adult mice clear lymphocytic choriomeningitis (LCM) virus. The T cell-mediated swelling that follows the local inoculation of virus allows parallel investigation of the infectious process and delayed-type hypersensitivity in one organ. A dose of $10^5$ mouse infectious units (IU) was optimal, and in all mouse strains tested foot swelling commenced 6 days after injection, with maximal response on days 7 and 8. When mice were sensitized by intravenous (i.v.) infection and challenged locally with infectious virus, the extent of swelling depended on both doses of virus and was most extensive when the interval between primary inoculation and local elicitation was 10 days. The rates of replication of the virus and its clearance were similar in the feet of mice of four strains tested, varying with regard to LCM virus-specific cell-mediated immunity. In CBA/J mice, virus elimination from the foot was followed for a longer time period and was incomplete up to 100 days after infection. A protocol for determining adoptive immunization was established; local inoculation of $10^8$ IU was followed 22 h later by i.v. infusion of $1 \times 10^8$ unselected or $4 \times 10^7$ T cell-enriched cells from the spleens of syngeneic donors that had been infected i.v. 7 or 8 days previously with $10^3$ IU. The concentrations of virus in the recipients' feet began to decline 2 to 3 days thereafter. Adoptive immunization by local inoculation of immune spleen cells was less successful, apparently because the virus multiplied in transferred cells.

INTRODUCTION

In order to understand the mechanism by which the adult mouse controls an acute infection with lymphocytic choriomeningitis (LCM) virus, events in the spleen have been analysed (Lehmann-Grube et al., 1988). This organ was chosen because there is (i) rapid multiplication and rapid elimination of the virus, (ii) easy quantification of the locally generated cells that are of paramount importance for the elimination process, i.e. cytotoxic T lymphocytes (CTL), and (iii) a vigorous response to adoptive immunization.

Of course, general conclusions about the control of LCM virus in the whole mouse cannot be drawn from this organ alone. The investigation was therefore extended to other tissues that would contrast functionally as well as anatomically with the spleen, and yet exhibit at least some of the useful features mentioned above. In this communication, efforts to standardize the experimental conditions for using the foot as an alternative are described.

METHODS

Virus. The WE strain LCM virus (Rivers & Scott, 1936) was used after it had been plaque-purified three times. It was propagated in L cells with Eagle's minimum essential medium containing non-essential amino acids (Lockhart & Eagle, 1959), supplemented with 5% heat-inactivated calf serum and antibiotics. Infectivity was titrated either as p.f.u. on L cell monolayers or as 50% mouse infectious doses, and all values thus obtained were converted to

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mouse infectious units (IU), 1 IU corresponding numerically to 1.50% infectious dose and 0.1 p.f.u. (Lehmann-Grube et al., 1985).

Mice. All mice used in this study were female, age 8 to 12 weeks. CBA/J (CBA) mice were purchased from either Jackson Laboratories (Bar Harbor, Me., U.S.A.) or Bomholtgård Breeding and Research Centre (Ry, Denmark). AKR and DBA/1LacJ (DBA/1) mice came from Jackson Laboratories and C57BL/6J (B6) mice from Bomholtgård Breeding and Research Centre. C3H/HeHan (C3H) and NMRI colony-bred mice were obtained from Zentralinstitut für Versuchstierzucht (Hannover, F.R.G.). Before use the animals were kept for at least 2 weeks under conditions prevailing during the experiments.

Quantification of infectious virus in foot tissue. The mice were killed by cervical dislocation and feet were amputated just proximal to the ankle joint. They were weighed and homogenized with known volumes of cold Hanks' balanced salt solution supplemented with 1% heated calf serum and antibiotics by use of pre-chilled mortars and sand. The homogenates were cleared of debris by centrifugation at 4 °C and snap-frozen in sealed ampoules to be stored at −70 °C until titration. Three aliquots of each homogenate were stored in this manner, and the first titration was performed in L cell cultures. If the quantity of infectious virus in foot tissue was too low for the plaque assay, the titration was repeated with mice.

Measurement of delayed-type hypersensitivity (DTH). Virus in 0.03 ml of diluent was inoculated subcutaneously (s.c.) into the right hind footpads of mice. At intervals, the dorso-ventral thickness of the inoculated and the uninoculated contralateral feet was measured by use of dial calipers ('Oditest'; H.C. Kröplin, Schöfftern, F.R.G.), and swelling is expressed as the factor with which the thickness of the inoculated foot exceeded that of the uninoculated foot (Lehmann-Grube & Löhler, 1981).

Adoptive immunization. Donor mice were infected intravenously (i.v.) by inoculation of 10^3 IU of virus. After 7 or 8 days the spleens were dispersed and either left untreated or depleted of adhering cells by incubating them twice for 1 h at room temperature in flat-bottomed glass vessels; in other experiments the immune spleen cells were enriched for T lymphocytes by passage through nylon wool columns (Julius et al., 1973). They were counted as viable on the basis of trypan blue exclusion and defined numbers were inoculated i.v. into syngeneic recipients that had previously been infected with 10^6 IU s.c. into the sole of the foot; alternatively, the immune cells were inoculated directly into the foot. The time intervals between (i) infection of recipient mice and inoculation of cells and (ii) inoculation of cells and determination of virus titres are specified in the Results section.

RESULTS

Foot swelling

One property of the foot that makes it useful for the present purpose is the swelling reaction following local inoculation s.c. of the virus. First described by Hotchin (1962) and Roger (1963), it has been used extensively to measure LCM virus-specific DTH (Tosolini & Mims, 1971; Zinkernagel, 1976; Lehmann-Grube & Löhler, 1981; Zinkernagel et al., 1985; Thomsen et al., 1983; Thomsen & Marker, 1986; Leist et al., 1987). LCM virus-specific swelling of feet differs between mice of different strains (Tosolini & Mims, 1971; Lehmann-Grube & Löhler, 1981). In my experience the thickness of feet of mice of a particular strain is similar within experiments. In contrast, both the time to and the degree of maximum swelling may differ greatly between experiments, even when care is exercised to keep all conditions identical. A statistical analysis of data that had been obtained between the years 1977 and 1979 (Lehmann-Grube & Löhler, 1981) (Table 1) reveals that the mean values obtained in individual experiments differed significantly. Despite this additional source of error, the difference between the mean values of swellings of all AKR and all CBA mice was found to be highly significant, which confirms the previous conclusion (Lehmann-Grube & Löhler, 1981; Lehmann-Grube et al., 1985) that the LCM virus-specific DTH in these two H-2 k strains is not solely governed by the major histocompatibility gene complex (MHC). The reason for the diversity of the results in individual experiments is not known. The influence of variables such as age and sex has frequently been tested and found to be irrelevant; only the quantity of the locally inoculated virus affected the degree of swelling in mice of all strains examined, i.e. B6, CBA, DBA/1 and NMRI. Data obtained with B6 mice are presented in Fig. 1.

LCM virus-specific DTH as revealed by foot swelling was also determined by use of a different protocol. Mice were sensitized by i.v. infection and were subsequently challenged by intraplantar inoculation of virus. Several experiments were performed in which virus dose and time interval were varied, and the results may be summarized by stating that the local inoculation of 10^3 IU did not cause any measurable alterations in the feet of previously infected
LCM virus infection of the foot of the mouse

Fig. 1. Swelling of feet of B6 mice after intraplantar infection with different quantities of LCM virus (●, $10^7$ IU; ■, $10^5$ IU; ▲, $10^3$ IU; ●, $10^1$ IU). Swelling is expressed as the factor by which the thickness of the inoculated hind feet exceeded the thickness of the contralateral control feet. Data points denote the mean values of 10 mice.

Table 1. Statistical treatment (analysis of variance) of maximal swellings of feet of mice of different strains after intraplantar inoculation of $10^4$ IU of LCM virus measured over a period of 32 months*

<table>
<thead>
<tr>
<th>Strain of mouse</th>
<th>NMRI</th>
<th>AKR</th>
<th>B6</th>
<th>CBA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total number of observations ‡</td>
<td>330</td>
<td>57</td>
<td>79</td>
<td>244</td>
</tr>
<tr>
<td>Mean of total (for all experiments)</td>
<td>1.9402§</td>
<td>1.9098</td>
<td></td>
<td>1.7756</td>
</tr>
<tr>
<td>Standard deviation of total (for all experiments)</td>
<td>0.2658</td>
<td>0.1861</td>
<td>0.2887</td>
<td>0.1829</td>
</tr>
<tr>
<td>Number of experiments</td>
<td>19</td>
<td>4</td>
<td>5</td>
<td>16</td>
</tr>
<tr>
<td>F (value of F-test)</td>
<td>3.3658</td>
<td>4.9752</td>
<td>5.5619</td>
<td>5.4946</td>
</tr>
<tr>
<td>F (F-test significance)</td>
<td>&lt;0.0001</td>
<td>&lt;0.005</td>
<td>&lt;0.001</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

* Data from Lehmann-Grube & Löhler (1981).
† All mice were 6 to 14 week old females and (for each strain) obtained from one source.
‡ Made throughout by one person.
§ Factor of swelling obtained by dividing the dorso-ventral thickness of the inoculated (right) foot by the corresponding measure of the contralateral (control) foot of the same mouse.
|| Difference between AKR and CBA mice $P < 0.0001$.

mice, whereas challenge with $10^5$ IU produced moderate swellings. Maximal local reactions were observed 24 h after inoculation of $10^7$ IU with a time interval between sensitization and elicitation of 10 days. Under these conditions, in B6 mice 1.7-fold increases in thickness were measured.

Replication of LCM virus in the foot of the mouse

Various quantities of virus were inoculated into the hind feet of B6 mice and the infectious titres were determined for 7 days (Table 2). After injection of 10 IU, replication commenced irregularly and the final virus concentrations varied considerably. The rates of replication
Fig. 2. Replication of LCM virus in and elimination from the feet of mice of four strains (●, CBA; ■, AKR; ▲, B6; ◆, DBA/1) after intraplantar infection. After subcutaneous inoculation of 10⁵ IU into right hind feet, they were homogenized and the virus was titrated. Data points and vertical bars denote the mean values of virus concentration and standard errors in groups of four mice.

Table 2. Replication of LCM virus in feet of B6 mice after intraplantar inoculation of various amounts of virus

<table>
<thead>
<tr>
<th>Time after inoculation (days)</th>
<th>IU inoculated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10¹</td>
</tr>
<tr>
<td>1</td>
<td>&lt;5 x 10²*</td>
</tr>
<tr>
<td>2</td>
<td>&lt;5 x 10²</td>
</tr>
<tr>
<td>3</td>
<td>~4.5 x 10³</td>
</tr>
<tr>
<td>4</td>
<td>9.5 (± 8.3) x 10⁵</td>
</tr>
<tr>
<td>5</td>
<td>3.1 (± 2.4) x 10⁵</td>
</tr>
<tr>
<td>6</td>
<td>3.9 (± 1.5) x 10⁵</td>
</tr>
<tr>
<td>7</td>
<td>1.9 (± 0.7) x 10⁶</td>
</tr>
</tbody>
</table>

* Mean (± standard error, where indicated) IU/g of foot tissue in four mice.

Table 2 differed after injection of 10³, 10⁵ or 10⁷ IU, and maxima were attained earlier with the higher doses. In further work, 10⁵ IU were usually employed.

It has previously been shown that replication of LCM virus in and elimination from spleens were similar in mice of four strains that varied with respect to LCM virus-specific cell-mediated immunity (Lehmann-Grube et al., 1985). The virus concentrations in the feet of mice of these strains were followed and only minor differences were found (Fig. 2). In these and other experiments, after an initial rapid drop of 99% to 99.9%, level values were maintained. In CBA mice the observation period was extended to 100 days after intraplantar infection, at which time more than 10⁴ IU/g of foot tissue were still present.

Spread of virus from the foot to lymphatic tissues

After the primary intraplantar inoculation of 10⁵ or 10⁷ IU, swelling was always obvious 6 days later, indicating that activated T lymphocytes were present in a tissue that normally contains few immunologically competent cells; thus, sensitization must be a rapid process. This probably takes place in the lymphoid organs, and 24 h after inoculation s.c. of 10⁵ IU the virus was readily demonstrable in spleen and/or lymph nodes. One of two similar experiments with essentially identical results is presented in Table 3.
Adoptive immunization

In general, DTH can be transferred to naive recipients with cells, following concomitant or sequential inoculation of antigen and immune cells into one site, such as the foot or ear. Virus, and subsequently spleen cells from mice expressing peaks of both LCM virus-specific DTH and CTL activities (day 7 or day 8 immune cells), were inoculated into a hind foot. Prompted by the report of Marchal et al. (1982) that DTH could be transferred locally by one T lymphocyte, knowing that LCM virus-specific DTH is transferred by K- and/or D-restricted T lymphocytes (Zinkernagel, 1976), which are high in number among the spleen cells of mice undergoing acute infection (Moskophidis et al., 1987a) and, furthermore, knowing that LCM virus in the mouse is controlled by the same subclass of T lymphocytes (Moskophidis et al., 1987b), preliminary experiments were performed with relatively low numbers of immune cells; these were consistently negative and caused neither swelling nor accelerated reduction of the infectious titre. It was found that at least $5 \times 10^7$ immune spleen cells had to be inoculated locally and 3 days had to elapse in order to reduce infectivity to a relevant degree (Fig. 3).

Six h after inoculation of this large number of cells, the feet were similarly enlarged whether immune or normal cells had been employed and whether the feet had received virus or were uninfected. However, after a further 18 h swelling was more extensive after the inoculation of virus and immune cells than after the inoculation of immune cells without virus or normal cells with and without virus (data not shown); similar results had been reported by Tosolini & Mims (1971).

As the data in Fig. 3 show, the reduction of infectious virus in the foot was comparable whether $5 \times 10^7$ immune spleen cells were inoculated locally or i.v. As suggested by the similar results of two experiments, of which one is presented in Fig. 4, the poor performance of the locally applied immune cells was apparent rather than real and resulted from multiplication of the virus in the inoculated cells themselves; when normal cells were injected, virus replication in the foot was greatly enhanced.
Fig. 4. The effect of local inoculation of normal and immune spleen cells on replication of virus in and elimination from feet of B6 mice. Donor mice were infected i.v. with $10^3$ IU. After 8 days their spleen cells were incubated twice for 1 h at room temperature in flat-bottomed glass vessels, counted as viable on the basis of trypan blue exclusion, and inoculated ($\bullet$, $5 \times 10^7$ and $\mathbb{R}$, $5 \times 10^6$ cells) into the feet of recipient mice that had been infected 22 h previously by inoculation s.c. with $10^4$ IU into the right hind feet. Other infected mice received similarly treated spleen cells from normal mice, ($\mathbb{R}$, $5 \times 10^7$ and $\mathbb{B}$, $5 \times 10^6$ cells) and control mice (I) did not receive any cells. At intervals, virus concentrations were determined; columns and bars represent means and standard errors, respectively, from five mice.

Fig. 5. Time relationship between swelling in and virus elimination from hind feet. At intervals after inoculation s.c. with $10^5$ IU into the right hind feet of CBA mice, their thickness was measured. Immediately thereafter, the feet were homogenized and the virus was titrated. Columns and data points denote means of virus concentration and foot swelling, respectively, in groups of five mice, and vertical bars signify standard errors.

Table 3. Spread of LCM virus to lymphatic tissues after intraplantar inoculation of $10^5$ IU

<table>
<thead>
<tr>
<th>Time after infection (days)</th>
<th>Mouse</th>
<th>Foot</th>
<th>Ipsilateral lymph nodes*</th>
<th>Contralateral lymph nodes*</th>
<th>Spleen</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>a</td>
<td>$8.8 \times 10^4$</td>
<td>$3.3 \times 10^7$</td>
<td>$&lt;2 \times 10^3$</td>
<td>$2.7 \times 10^3$</td>
</tr>
<tr>
<td></td>
<td>b</td>
<td>$8.8 \times 10^4$</td>
<td>$1.9 \times 10^7$</td>
<td>$&lt;2 \times 10^3$</td>
<td>$&lt;2 \times 10^3$</td>
</tr>
<tr>
<td>2</td>
<td>a</td>
<td>$7.6 \times 10^5$</td>
<td>$&lt;2 \times 10^3$</td>
<td>$&lt;2 \times 10^3$</td>
<td>$7.9 \times 10^4$</td>
</tr>
<tr>
<td></td>
<td>b</td>
<td>$1.2 \times 10^6$</td>
<td>$1.9 \times 10^8$</td>
<td>$2.3 \times 10^4$</td>
<td>$1.1 \times 10^5$</td>
</tr>
<tr>
<td>3</td>
<td>a</td>
<td>$5.1 \times 10^6$</td>
<td>$3.5 \times 10^8$</td>
<td>$&lt;2 \times 10^3$</td>
<td>$1.7 \times 10^7$</td>
</tr>
<tr>
<td></td>
<td>b</td>
<td>$4.2 \times 10^6$</td>
<td>$8.2 \times 10^8$</td>
<td>$&lt;2 \times 10^3$</td>
<td>$8.8 \times 10^6$</td>
</tr>
<tr>
<td>4</td>
<td>a</td>
<td>$1.6 \times 10^6$</td>
<td>$8.8 \times 10^7$</td>
<td>$6.9 \times 10^7$</td>
<td>$5.1 \times 10^8$</td>
</tr>
<tr>
<td></td>
<td>b</td>
<td>$4.0 \times 10^6$</td>
<td>$1.3 \times 10^8$</td>
<td>$1.2 \times 10^8$</td>
<td>$1.0 \times 10^9$</td>
</tr>
</tbody>
</table>

* Popliteal and inguinal lymph nodes.
† IU/g of tissue of individual mice.

No difficulties were experienced in adoptively immunizing mice by inoculation of cells i.v. In the protocol finally adopted, recipients were locally infected with $10^5$ IU and transfused i.v. 22 h later with $10^8$ unselected or $4 \times 10^7$ T lymphocyte-enriched spleen cells obtained 7 or 8 days after the immunizing infections of donor mice. Under these conditions, viral infectivity began to decline (relative to control mice) 2 to 3 days after cell transfer, and was reduced by more than 95% within 5 days. Differences between C3H, B6 and CBA mice were not apparent.

Time relationship between swelling and virus elimination

In the spleen, virus elimination commences at a time when CTL activity becomes demonstrable (Marker & Volkert, 1973; Lehmann-Grube et al., 1985). In the foot, the time
relationship between virus clearance and local expression of cell-mediated immunity is different, and in a number of experiments the virus concentration began to decline only when the expression of DTH had become maximal (Fig. 5). The delay in virus elimination (relative to swelling) was even more obvious after adoptive immunization (Fig. 6).

**DISCUSSION**

Besides LCM virus, numerous viruses have been shown to induce DTH in experimental animals and man, as revealed by inflammatory skin reactions developing after local application of antigen, which suggests that this is a general phenomenon and probably of considerable relevance in the termination of infection or for immunopathological phenomena in virus diseases.

DTH is generally assumed to be an expression of the interaction of at least two types of activated cells, antigen-specific T lymphocytes and antigen-non-specific mononuclear phagocytes. As demonstrated by Zinkernagel (1976), the T lymphocytes that mediate LCM virus-specific DTH are restricted by MHC-encoded molecules of class I (the cytotoxic/suppressive subtype), which are also instrumental in eliminating virus from organs of acutely infected adult mice (Zinkernagel & Welsh, 1976; Moskophidis et al., 1987b). Mononuclear phagocytes, however, do not appear to participate in clearance of the agent (Lehmann-Grube et al., 1987), which makes it unlikely that DTH is of relevance in the mechanism. This conclusion is supported by other findings. Thus replication of the virus in the feet of mice of different strains is not measurably influenced by the degree to which they respond with a local DTH reaction; similar observations had been reported by Tosolini & Mims (1971). Here it is shown that the same is true with regard to virus elimination; there are also kinetic differences between swelling and virus clearance.

In contrast to other mouse tissues from which the virus is rapidly and apparently completely eliminated (e.g. spleen and liver) (Lehmann-Grube et al., 1985; Moskophidis et al., 1987b; F. Lehmann-Grube, unpublished results), infectivity drops by 99% to 99.9% in the foot by day 10 after infection, but the subsequent elimination is slow and virus remains detectable for at least
100 days. It is known that after acute infection low concentrations of LCM virus may remain for long periods in certain murine tissues (Volkert & Hannover Larsen, 1965); the foot appears to be one such organ.

It proved difficult to demonstrate adoptive immunization by locally inoculating immune cells, and accelerated elimination of virus from the foot required the injection of at least $5 \times 10^6$ day 8 immune spleen cells. Since there is reason to assume that approximately 10% were LCM virus-specific CTL or their activated precursors (Moskophidis et al., 1987a), it may be concluded that as many as $5 \times 10^5$ of these cells, although applied locally, failed to clear the virus effectively. This contrasts with the spleen, in which multiplication of LCM virus was affected by the presence of as few as 200 specifically activated CTL (Lehmann-Grube et al., 1988). The present findings suggest that the foot is different in this respect because of virus multiplication in the locally injected cells, thereby resulting in a partial reversal of the antiviral activity of the CTL.

The LCM virus-specific DTH reaction is primary in the sense that a single footpad inoculation of virus sensitizes the animal and elicits DTH. In this regard it differs from most other examples of virus-specific DTH in which sensitization and challenge are separated by time intervals of a few days. However, i.v. infection followed by local injection of virus also resulted in swelling, which was extensive when the challenge inoculum was applied 10 days after sensitization. This is 2 to 3 days later than the occurrence of maximal swelling after intraplantar inoculation of the virus into mice not previously sensitized, which is not easily explained, although a similar finding has been reported by Tosolini & Mims (1971).

The aim of this work was to find a tissue that would supplement the spleen for analysis of the mechanism by which a higher organism controls a virus infection. The virus does not multiply as rapidly in the foot and is not as efficiently eliminated; also, adoptive immunization is not as vigorous as it is in the spleen. There is also the methodological problem of reproducibility of the local response. The observations reported here indicate that within experiments quantitative evaluation is possible, but between experiments the variation of data is extensive and there is a need for caution when findings obtained at different times or in different places are to be compared. The swelling following the intraplantar inoculation of LCM virus results predominantly from the accompanying oedema rather than from the number of locally accumulated cells (J. Löhler, personal communication); thus, the thickness of the foot is a priori not a reliable measure for DTH and, by implication, the animal's cell-mediated immunity. Despite these shortcomings, studies of the foot should provide, perhaps better than studies of the spleen, information as to the kind of cells that are involved in virus clearance; in particular, whether cooperation of T lymphocytes with mononuclear phagocytes is required or whether some other T cell-mediated mechanism not involving macrophages and related elements is essential.

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