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# CELL-MEDIATED IMMUNITY IN FLAVIVIRUS INFECTIONS. I. INDUCTION OF CYTOTOXIC T LYMPHOCYTES IN MICE BY AN ATTENUATED VIRUS FROM THE TICK-BORNE ENCEPHALITIS COMPLEX AND ITS GROUP-REACTIVE CHARACTER

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*Summary.* — The lytic activity of splenocytes from C3H mice immunized with a highly attenuated line of Langat virus [tick-borne encephalitis (TBE) complex] was determined by the in vitro $^{51}Cr$ release assay on TBE virus (western subtype)-infected target L929 cells. After the spleen cell suspensions were depleted of T cells with anti-mouse theta serum, or of B cells with anti-mouse immunoglobulin, the cytotoxic effect was found dependent on the presence of T lymphocytes. During immunization with Langat virus, cytotoxic T lymphocytes were generated, specific for both the foreign virus-specified antigen and the self component of the host system studied, coded by the K allele of the H-2 complex. The peak of T lymphocyte cytotoxic response was attained on the 6th day after administration of the live virus. T lymphocytes from mice, single-shot immunized with a flavivirus, displayed distinct cross-reactive lysis when studied on target cells infected with other flaviviruses but not on cells infected with an alphavirus.

*Key words: flavivirus infections*; *H-2 histocompatibility*; *cytotoxic T lymphocytes*; $^{51}Cr$ *release assay*; *cross-reactive lysis*

## *Introduction*

As shown previously, the highly attenuated E5 "14" line derived from the naturally attenuated Langat virus, belonging to the tick-borne encephalitis (TBE) complex of viruses, induces in mice a markedly expressed resistance against virulent challenge with other members of the TBE complex (Mayer, 1975). The extremely low levels of specific virus-neutralizing antibodies and the very effective clearance of the challenging virus suggested that also the cell-mediated component of the immune response (CMI) may participate in the state of resistance elicited by the live low virulent virus.

Specific recognition of TBE viral antigens by lymphocytes from TBE convalescents and from mice experimentally infected with attenuated Langat E5 "14" virus was definitely proved by the capillary method of lymphocyte migration inhibition (Mayer *et al.*, 1976; Gajdošová *et al.*, 1978).

In the present paper we report the results of investigations on the cytotoxic response of T lymphocytes generated in mice by the administration of attenuated live Langat virus. For the evaluation of the $^{51}Cr$ release from target cells by cytotoxic effector cells, a syngeneic system, i.e. inbred C3H mice and L929 mouse fibroblast cells were used, taking into account the importance of H-2 complex gene products for the recognition by thymus-dependent lymphocytes from primed mice of virus-associated antigen(s) present on the surface of infected target cells.

A preliminary report on some of the results has been published (Gajdošová *et al.*, 1980).

## *Materials and Methods*

*Mice.* Inbred male C57BL/10 (H-$2^b$), DBA-2/J (H-$2^d$) and C3H/Cbi/BOM (H-$2^k$) mice were obtained from the Šumice breeding farm. The animals (usually 4—5 weeks old) were given one intravenous (i.v.) dose of Langat E5 "14" virus ($10^3$ PFU/0.1 ml). Control animals were given one i.v. dose of appropriately diluted chick embryo suspension.

*Viruses. Langat virus* isolate "14" derived from the Langat E5 virus strain (Mayer, 1975) was used after it had undergone 8 passages in 7 days SPF chick embryos. One ml of a 10 % chick embryo suspension contained 6.2 dex newborn mouse i.c. $LD_{50}$/ml, corresponding to $10^4$ PFU on pig kidney (PS line) cell monolayers. Strain "204" of *TBE virus*, western subtype (Grešíková, 1975), had undergone 10 suckling mouse brain passages. The stock virus suspension used had a titre of 8.2 dex i.c. $LD_{50}$/ml ($4.1 \times 10^8$ PFU on PS cell monolayers). The 17D variant of *Yellow Fever* (*YF*) virus originating from a commercial vaccine prepared in chick embryo cells (Burroughs Wellcome and Co; London; Yellow Fever vaccine B. P., Leukosis free, lot 4512) was used after three i.c. passages in newborn SPF mice. The stock virus suspension had a titre of $1.8 \times 10^6$ PFU/ml. *West Nile* (*WN*) *virus* strain "99" isolated from mosquitoes in Wertern Slovakia (Labuda *et al.*, 1974) had undergone 12 suckling mouse brain passages. The stock virus suspension used had a titre of 7.5 dex i.c. $LD_{50}$/ml ($1.1 \times 10^8$ PFU on PS cell monolayers). *Dengue type 1* (*D 1*) *virus*: the prototype Hawaiian strain obtained from the WHO Collaborating Centre for Arboviruses, Institute of Virology, Slovak Academy of Sciences, Bratislava, had undergone nine suckling mouse brain passages. The stock virus suspension used had a titre of 7.5 dex i.c. $LD_{50}$/ml. *Sindbis virus* obtained from the WHO Collaborating Centre for Arboviruses, Institute of Virology, Slovak Academy of Sciences, Bratislava, had undergone two suckling mouse brain passages and a high number of passages in chick embryo cell cultures. The stock virus suspension used had a titre of 8.2 dex i.c. $LD_{50}$/ml.

*Cytotoxic assay. Effector cells.* On the sixth day after infection, the mice were killed by cervical dislocation and their splenocytes used as effectors for the T-lymphocyte cytotoxic test. Splenocytes were prepared as described (Gajdošová and Mayer, 1978) from pools of 8—10 spleens. *Target cells.* The fibroblast cell line L929 (L cells) derived from C3H mice, was used as a source of target cells throughout. The cells were grown in basal Eagle's medium (BEM) with 5 % foetal calf serum (FCS). After 48 hr of incubation at 37 °C, tube cultures were infected with the "204" strain of TBE virus or another flavivirus (WN, YF, D 1) at an input multiplicity of 2 PFU per cell. *Determination of $^{51}Cr$ release.* Monolayers of infected or unifected target cells at a density of $5 \times 10^5$/ /tube were labelled with 0.18 MBq $^{51}Cr$ ($Na_2{}^{51}CrO_4$; specific activity 3.7—14.8 MBq/mg of chromium; Akademie der Wissenschaften der DDR, Zentralinstitut für Kernforschung, Abteilung für radioaktive Präparate, Dresden) per tube. After 60 min at 37 °C, the cells were washed 3 times with phosphate buffered saline (PBS). The PBS was then removed and immune or normal spleen cells were added in 1 ml of L-15 medium (Leibovitz, 1963) with 10 % FCS. Spleen: target cell ratios of 100 : 1 were routinely used (i.e. $5 \times 10^7$ spleen cells and $5 \times 10^5$ target cells per tube). After further incubation at 37 °C (usually 18 hr) all tubes were centrifuged at 400 rev/min for 15 min and the supernatants were transferred to separate tubes. Then the activities of supernatant, cells and lysate were measured. $^{51}Cr$ release from target cells in each tube was calculated according to the formula proposed by Zinkernagel and Doherty (1974):

$$\%\,{}^{51}Cr \text{ release} = \frac{\text{count/min in supernatant} \times 100}{\text{count/min in supernatant} + \text{count/min in cells}} \times \frac{100}{\%\ \text{lysis}}$$

**Table 1. Cytotoxic effect of spleen cells from various strains of mice against TBE virus-infected target cells**

| Effector cells from the mouse strain* | MHC (H-2 region) | | % $^{51}$Cr release (± S.D.) from target cells** | |
|---|---|---|---|---|
| | K | D | uninfected | infected |
| C57BL/10 | b | b | 24.7 ± 0.9 | 28.2 ± 0.8 |
| DBA-2/J | d | d | 20.8 ± 1.1 | 25.4 ± 0.6 |
| C3H/Cbi/BOM | k | k | 21.7 ± 1.5 | 79.3 ± 1.2 |

* Splenocytes were tested 6 days after intravenous immunization.
** L929 cells (H-2$^k$) infected with TBE virus.
MHC — major histocompatibility complex; alleles of respective H-2 regions are designated by small letters.

% of lysis was determined after freezing and thawing target cells 3 times. The amounts of radioisotope expressed in count/min were measured in triplicate in a gamma radiation counter (Tesla 54). Standard deviations (S.D.) of experimental samples were calculated. Different groups were compared for statistical significance by the two-tailed Student's t-test. Spontaneous $^{51}$Cr release was measured from cultures of target cells alone in L-15 medium.

*Rabbit anti-mouse theta serum* (*AΘS*) was prepared as described by Golub (1971). New Zealand white rabbits were given three intramuscular injections of whole C3H mouse brain suspension homogenized in complete Freund's adjuvant at two-week intervals and bled 2 weeks after the last injection.

*Swine anti-mouse immunoglobulin* (Ig) was kindly provided in a lyophilized form by Dr. K. Hrudková, Institute of Sera and Vaccines, Prague. After absorption of reconstituted Ig with erythrocytes, liver cells and either thymocytes or bone marrow cells (Goscicka *et al.*, 1976), the sera were inactivated at 56 °C for 30 min and stored at −20 °C. Cytotoxic specificity of these sera was determined on isolated thymus and bone marrow cells in the presence of guinea pig serum diluted 1 : 5 as a source of complement (C). The guinea pig serum was absorbed with Difco Noble agar before use (Cohen and Schlesinger, 1971).

*Cytotoxicity of antisera* was determined by the trypan blue dye exclusion test after incubation at 37 °C for 30 min. The AΘS possessed the ability to kill more than 90 % of thymocytes in a 1 : 10 dilution in the presence of C and less than 5 % of bone marrow cells. The anti-mouse Ig was used at a dilution of 1 : 10, which caused 90 % and about 5 % cytotoxicity with bone marrow cells and thymocytes, respectively.

*Depletion of T and B lymphocytes* was assayed by the method of Nagarkatti *et al.* (1978). Treated spleen cells were suspended in 0.5 ml of a 1 : 10 dilution of AΘS, or Ig in BEM and 0.5 ml of a 1 : 5 dilution of guinea pig. C. The mixture was incubated at 37 °C for 1 hr, washed and suspended in L-15 medium with 5 % FCS, counted for viable cells and used in $^{51}$Cr release cytotoxic assay.

Normal rabbit and normal swine sera were used in a dilution of 1 : 10 after absorption like AΘS or Ig.

## *Results*

### *H-2 restriction of cytotoxic effector cell activity*

To determine whether the response was restricted to target cells histocompatible with the immunized mice, we tested splenocytes from C57BL/10, DBA-2/J and C3H/Cbi/BOM immunized mice on virus-infected target cells. A cytotoxic response was demonstrable only on the syngeneic virus-infected target cells (Table 1).

*Specificity of cytotoxic activity of Langat virus immune spleen cells against TBE virus-infected L929 cells*

The levels of cytotoxicity determined by $^{51}Cr$ release are shown in Fig. 1. Group 1 presents spontaneous $^{51}Cr$ release from target cells alone (uninfected cells 23.50% and infected cells 21.57%). With effector cells from Langat

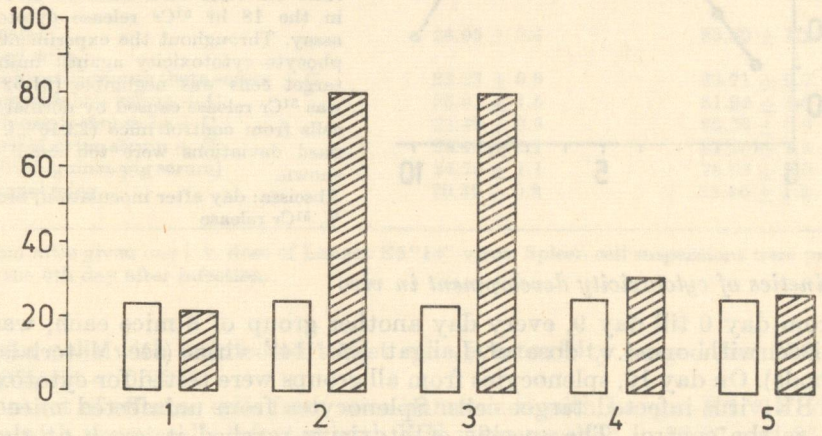


**Fig. 1.**

Specific immune lysis of TBE virus-infected L cells by spleen cells from C3H mice immunized intravenously with Langat E5"14" virus
Empty columns: unifected target cells; shaded columns: infected target cells
1 — target cells in medium L-15
2 — target cells + effector cells from 25—30 g mice immunized with virus
3 — target cells + effector cells from 10—12 g mice immunized with virus
4 — target cells + effector cells from uninfected mice
5 — target cells + effector cells from mice immunized with chick embryo suspension
Each value represents the mean of three measurements (individual tubes). The spleen cells were tested at a 100 : 1 ratio.
Ordinate: % $^{51}Cr$ release.

virus-immunized mice, we observed 80.20 and 80.07% of $^{51}Cr$ release in groups 2 and 3, in which older and younger animals, respectively, were used. The immune answer of animals in the age range investigated was thus similar. The results in groups 2 and 3 are highly significant ($P < 0.001$) when compared with results of $^{51}Cr$ release from uninfected target cells. In group 4 (effector cells: splenocytes from control, uninfected mice) and group 5 (spleen cells from mice given chick embryo suspension only), the results were similar to those observed in group 1.

Cytotoxicity thus appeared to be virus antigen-specific in that Langat virus-immune spleen cells killed TBE-infected L cells, but not uninfected L cells, whereas spleen cells from normal uninfected mice, or mice immunized with chick embryo suspension did not significantly damage TBE-infected L cells.

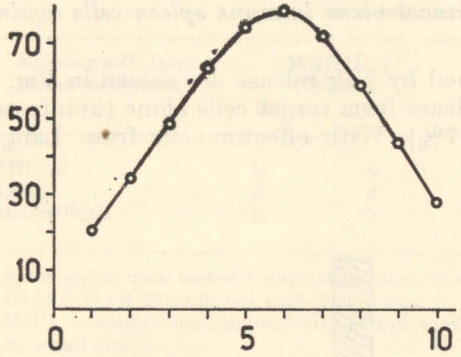


**Fig. 2.**
Kinetics of cytotoxicity against TBE virus-infected L cells by splenocytes from mice inoculated i. v. with the attenuated Langat E5"14" virus
The spleen cells were tested at a 100 : 1 ratio on TBE virus-infected L929 cells in the 18 hr $^{51}Cr$ release cytotoxicity assay. Throughout the experiment, lymphocyte cytotoxicity against uninfected target cells was negligible (21.52%) as was $^{51}Cr$ release caused by normal spleen cells from control mice (23.46%). Standard deviations were too small to be shown.
Abscissa: day after inoculation; ordinate: % $^{51}Cr$ release

*Kinetics of cytotoxicity development in vivo*

From day 0 till day 9, every day another group of 8 mice each, was immunized with one i.v. dose of Langat E5 "14" virus (see Materials and Methods). On day 10, splenocytes from all groups were tested for cytotoxicity on TBE virus-infected target cells. Splenocytes from uninfected mice were used as the control. The specific cytotoxicity reached its peak at the 6th day and then declined to a low level by day 10 (Fig. 2).

*Effect on T-lymphocyte cytotoxicity of anti-mouse theta serum and anti-mouse immunoglobulin in the presence of complement*

The results are summarized in Table 2. Treatment of immune spleen cells with rabbit anti-mouse theta serum and C to deplete specifically the T-cell population substantially reduced the effector cell function (23.71%). In addition, depletion of B cells with swine anti-mouse Ig and C did not decrease cytotoxicity (80.26%) as compared with cytotoxicity of untreated immune spleen cells (83.50%). These data indicate that it is primarily the T-cell population of the spleen that effects cytotoxicity.

Incubation of spleen cells from immunized mice with normal rabbit serum and C was without demonstrable effect on T-cell cytotoxicity (81.92%), as was incubation of immune spleen cells with normal swine serum and C (82.25%).

*Cross-reactive cytotoxic effect of T cells*

Individual groups of C3H mice were given i.v. $10^3$ PFU of Langat E5 "14", YF 17D, WN or D 1 flavivirus or Sindbis alphavirus. Six days after infection their splenocytes were used as effector cells in the cytotoxic assay by $^{51}Cr$ release from target cells infected with the "204" strain of TBE virus, or with WN, YF, or D 1 virus. The results are summarized in Table 3.

After contact of effector cells from mice infected with WN, YF, Langat E5 "14", or D 1 virus with target cells infected with the "204" strain of TBE virus, cytotoxicity ranged from 78.39 to 86.10; after contact of the same

**Table 2. Per cent specific $^{51}Cr$ release from TBE virus-infected L cells by spleen cells treated with rabbit anti-mouse theta serum, or with swine anti-mouse immunoglobulin in presence of complement**

| Spleen cell population as effector* | % $^{51}Cr$ release (±S. D.) from target cells uninfected | infected |
|---|---|---|
| Untreated | 24.05 ± 0.4 | 83.50 ± 1.2 |
| Treated with | | |
| Rabbit anti-mouse theta serum + C | 22.23 ± 0.9 | 23.71 ± 0.7 |
| Normal rabbit serum + C | 25.91 ± 1.5 | 81.92 ± 1.6 |
| Swine anti-mouse Ig + C | 23.49 ± 0.6 | 80.26 ± 0.9 |
| Normal swine serum + C | 23.45 ± 2.1 | 82.25 ± 1.3 |
| C (fresh guinea pig serum) | 24.78 ± 1.1 | 79.93 ± 2.0 |
| Normal mice | 20.32 ± 0.8 | 23.46 ± 1.2 |

* From mice given one i. v. dose of Langat E5 "14" virus. Spleen cell suspensions were prepared on the 6th day after infection.

effector cells with uninfected target cells cytotoxicity ranged from 20.49 to 26.10% ($P < 0.001$).

Contact of effector cells from mice immunized with Langat E5 "14" or YF virus with target cells infected with WN virus resulted in 83.27 and 85.07% cytotoxicity, while after the contact of the same effector cells with uninfected target cells we observed a 21.93 and 26.10% cytotoxicity ($P < 0.001$).

With effector cells from D 1 virus-infected mice, the cytotoxicity was 79.97% for target cells infected with the same virus (D 1) and 25.17% for unifected target cells ($P < 0.001$).

With effector cells from Sindbis virus-infected mice, we found no significant cytotoxicity (33.77%) for target cells infected with the "204" strain of TBE virus as compared with uninfected target cells (28.15%).

**Table 3. Cross-reactive cytotoxic activity of effector T cells from mice immunized with various flaviviruses on target L929 cells infected with TBE, WN, YF and D1 viruses**

| Effector T cells from mice immunized with virus | % $^{51}Cr$ release (mean ± S. D.) from target L929 cells Uninfected | Infected with virus TBE "204" | WN | YF | D1 |
|---|---|---|---|---|---|
| WN | 20.49 ± 1.5 | 78.39 ± 1.5 | — | — | — |
| YF 17D | 21.93 ± 1.0 | 82.52 ± 1.6 | 85.07 ± 1.6 | — | — |
| D 1 | 25.17 ± 0.7 | 80.99 ± 1.1 | — | — | 79.97 ± 1.1 |
| Langat E5 "14" | 26.10 ± 1.8 | 86.10 ± 2.7 | 83.27 ± 2.3 | 87.15 ± 1.5 | — |
| Sindbis (*Alphavirus*) | 28.15 ± 1.6 | 33.77 ± 2.2 | — | — | — |
| Uninfected mice | 21.22 ± 0.9 | 24.13 ± 0.8 | 23.33 ± 1.6 | — | — |
| Spont. $^{51}Cr$ release from target cells | 23.72 ± 1.3 | 23.28 ± 1.2 | 24.85 ± 1.4 | — | — |

— = Not done.

*Discussion*

We observed in our earlier studies that, after specific in vivo stimulation of murine splenic lymphocytes and after their in vitro contact with the given antigen (TBE virus), the migratory activity of these cells was inhibited (Gajdošová and Mayer, 1978). This suggested the production of a lymphokine (lymphocyte migration inhibition factor) by sensitized T cells and, accordingly, the involvement of CMI in the specific resistance elicited by abortive Langat virus infection. We never demonstrated the presence of immunizing virus in the blood or organs from primed mice.

The present study, the cytolytic activity against TBE virus-infected target cells was demonstrated in murine lymphocytes after i.v. administration of attenuated Langat E5 "14" virus to these animals. Target cell lysis was restricted by the H-2 complex in that only C3H (H-$2^k$) effector cells strongly lysed infected L929 cells (H-$2^k$) as could be expected in this syngeneic system used. These results are in accordance with findings that sharing of alleles at certain loci of the H-2 region (on chromosome 17 of the mouse) is essential for optimal interaction of lymphocytes with target cells in cytotoxic killing by T lymphocytes (Gardner *et al.*, 1975; Gordon *et al.*, 1975; Blanden *et al.*, 1975; Doherty and Zinkernagel, 1975; Zinkernagel and Doherty, 1975).

We measured cytotoxicity by $^{51}Cr$ release assay, which is based on the observation that the radioactive chromate ($^{51}CrO_4^{2-}$) ion, following diffusion through the cell membrane, is retained in the cytoplasm for a relatively extended period of time. Thus $^{51}Cr$ release from a labelled target cell into the supernatant fluid does not occur unless the cell membrane is sufficiently damaged. The isotopically labelled material that is released is not subsequently reincorporated into undamaged cells (Brunner *et al.*, 1976).

After intravenous infection of mice with Langat virus the cytotoxic activity of spleen cells rose to a peak on the 6th day after infection and then fell to a low level by day 10. The cytotoxicity elicited in our experiments by other viruses also reached a peak on the 6th day. A similar rise and fall of cytotoxic potency in spleen cells of mice immunized with attenuated ectromelia virus was observed by Gardner *et al.* (1974*a*).

Gardner *et al.* (1974*b*) demonstrated in the same system that treatment of immune cells with anti-theta ascitic fluid and C eliminated the cytotoxic activity of the final cell suspension (% $^{51}Cr$ release after treatment of spleen cells with anti-theta ascitic fluid and C was similar to % $^{51}Cr$ release from normal uninfected target cells).

Elimination of T cells from immune spleen cell population by treatment with anti-mouse theta serum and C abrogated cytotoxicity in our experiments. $^{51}Cr$ release after such treatment reached values approaching those obtained on normal uninfected target cells. Depletion of B cells with anti-mouse immunoglobulin and C did not decrease cytotoxicity thus indicating the participation of T cells in the cytotoxic assay also in the TBE virus-host system studied. We observed an ability of effector cells from mice immunized with a flavivirus to display an efficient cross-reactive cytotoxic activity on L

cells infected with the same or another flavivirus. The levels of cross-cytotoxicity reached, as demonstrated on target L929 cells, were higly significant as compared with uninfected target cells. The reason of this observation is unknown at present. One possibility to account for the cross-reactivity observed would be an exact demonstration of recognition by cytotoxic T lymphocytes of group-reactive determinants of the haemagglutinin (shared by members of the *Flavivirus* genus), presumably present on the infected target cells. The problem whether flavivirus infections generate two populations of cytotoxic effector cells (as suggested with influenza A viruses by Doherty *et al.*, 1977), one being specific for the infecting virus and the other being group-reactive, awaits further detailed biochemical and antigenic analysis of events governing the infectious cycle of flaviviruses and production of their structural proteins, as well as a better understanding of the nature of the T-cell receptor(s).

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