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STUDIES ON THE CYTOTOXIC EFFECT OF *IN VIVO* AND *IN VITRO* IMMUNIZED LYMPHOCYTES ON LIVER TARGET CELLS

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SUMMARY

Lymph node and spleen cells from mice immunized *in vivo* to allogeneic or syngeneic liver antigen are cytotoxic for syngeneic liver cells, but not for syngeneic fibroblasts or established liver cell cultures of allogeneic origin. The cytotoxic activity is mainly dependent on T-cell activity, but a non-T-cell-mediated cytotoxicity may also play a role. Lymphocytotoxicity is inhibited by preincubation of the lymphocytes with syngeneic liver antigen, but not with syngeneic kidney homogenate. The liver-specific lymphocytotoxicity corresponds to the *in vivo* function of lymphocytes in the development of experimental hepatitis. *In vitro* immunization of lymphocytes in a Mishell–Dutton culture system also induces liver-specific cytotoxicity. The results indicate that the natural tolerance to self antigens can be lost after *in vivo* as well as *in vitro* immunization. The induction of self-reactivity of lymphocytes in these experiments may be attributed to regulatory mechanisms of the immune reaction at a cellular level.

INTRODUCTION

Experimental hepatitis is induced in inbred mice by injection of allogeneic liver homogenate (Scheiffarth & Warnatz, 1967; Warnatz, 1969b) and can be transferred to syngeneic mice with lymphocytes, but not with serum (Warnatz *et al.*, 1967; Scheiffarth, Warnatz & Liebelt, 1968). Neonatally thymectomized mice do not develop the experimental hepatitis (Warnatz, Scheiffarth & Schmidt, 1967b). Incubation of lymph node and spleen cells from mice with experimental hepatitis with liver antigen induces an increased incorporation of [^{3}H]thymidine into nuclear DNA of lymphocytes in short-term cultures (Warnatz, 1969a). It was concluded from these results that the induction of experimental hepatitis is a T cell-dependent immune reaction.

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In the present experiments mouse lymphocytes were immunized to allogeneic or syngeneic liver antigen *in vivo*, and *in vitro* in a Mishell–Dutton system. The lymphocytes were examined for their cytotoxic effect on syngeneic or allogeneic liver cells. It was tested whether the lymphocytotoxicity was a T cell-dependent immune reaction and whether it is liver cell-specific. The findings of this study have to be discussed in the light of the new hypothesis on the mechanisms underlying autosensitization *in vivo* and *in vitro*.

MATERIALS AND METHODS

Animals

In the experiments inbred AKR mice and inbred C57Bl6 mice were used. The mice were from our own breeding colony.

Cell cultures

Liver target cells were cultured from livers of 3–4-week-old embryos of the AKR or of the C57Bl6 strain. The livers were removed under sterile conditions and cut into small pieces. They were trypsinized in 0·2% trypsin solution at 37°C for 10 min. 5×10^5 Isolated liver cells were cultured per millilitre of RPMI 1640 medium with 15% foetal bovine serum for 3 days. At that time the cells formed a complete monolayer. The cells were trypsinized and again cultivated in RPMI 1640 medium. After three passages the cell pattern was rather homogeneous. The cells were used in cytotoxicity assay. Besides the embryonal liver cells skin fibroblasts from adult AKR mice or C57Bl6 mice were prepared as target cells. In control experiments NCTC cells (established mouse liver cells, cultures obtained from Microbiological Associates) were used.

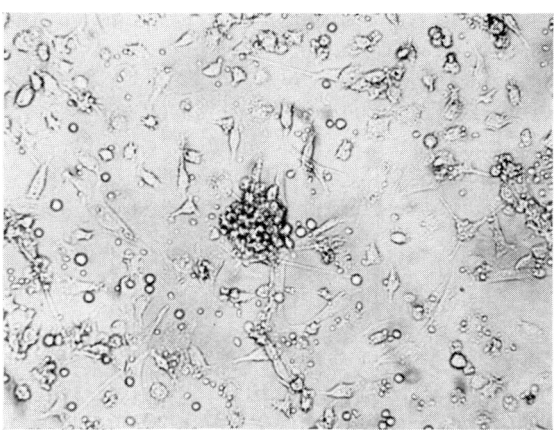

FIG. 1. Culture of embyronal liver cells from AKR mice incubated with syngeneic lymphocytes.

In vivo immunization of lymphocytes to allogeneic liver

Seventy-five AKR mice and seventy-five C57Bl6 mice were immunized with allogeneic liver homogenate. It was prepared from livers of adult AKR or C57Bl mice, respectively, by

homogenization with a Potter-Elvehjem homogenizer. The whole liver homogenate, diluted 1:9 with Hanks's solution (final protein content 100 mg/ml), was centrifuged for 10 min at 500 *g*; the supernatant was used for immunization of mice. It was mixed with complete Freund's adjuvant in a ratio of 1:1. 0.1 ml of the mixture corresponding to 5 mg of protein were injected into the footpads of the mice. Five further subcutaneous injections of liver homogenates were administered at weekly intervals. Ten days after the last injection the animals were killed and the livers were removed for histological examination.

Spleens and lymph nodes were collected and the lymphocytes were isolated from the organs by teasing with forceps. The lymphocytes were suspended in RPMI 1640 medium completed with glutamine, sodium pyruvate and a mixture of non-essential amino acids. For separation of T cells, lymphocytes were passed through a nylon wool column of 5 cm length and 1·2 cm diameter (Julius, Simpson & Herzenberg, 1973). The passing cells were washed from the nylon wool with completed RPMI 1640 medium with 2.5% foetal bovine serum. The cells from C57Bl6 mice were tested for T lymphoctyes by the percentage cytolysis after incubation with anti-θ serum and complement. The anti-θ serum was produced by immunization of AKR mice with C3H thymocytes for 3 months. Blood was obtained by puncture of the retro-orbital plexus; after clotting at room temperature serum was drawn off and stored at -70°C without further treatment. The cytotoxic titre of the anti-θ serum was determined as 1:5000. Non-T cells were prepared from unfractionated cells of C57Bl6 mice by treatment with anti-θ serum and complement.

In vitro immunization of lymph node and spleen cells

AKR mice or C57Bl6 mice were killed and lymph nodes and spleens removed. The cells isolated from the organs were suspended in Eagle's minimal essential medium. 2×10^7 Cells were cultured according to the modified technique of Mishell and Dutton (1967; Warnatz, Scheiffarth & Stiegler, 1974). For immunization to liver antigen 5×10^5 liver cells isolated from the livers of C57Bl6 mice by trypsinization were added to the cultures. In control experiments lymphocytes were cultured in the absence of liver cells. The cultures were incubated at 37°C in a humidified atmosphere of 7% O_2, 10% CO_2 and 83% N_2 on a rocker platform. The cultures were fed each day with 100 μl of nutritional mixture and 30 μl of foetal bovine serum. The cells were harvested on day 4. Cell suspensions pooled from four Petri dishes were centrifuged at 1000 rev/min for 10 min at 4°C. The counts of viable cells were estimated by the trypan blue exclusion test. After 4 days cultivation more than 40% of the incubated cells were viable.

Microcytotoxicity test

The test was performed according to the instructions described by Takasugi and Klein (1970), modified by Hellström *et al.* (1971). As target cells, liver cells from AKR mice and C57Bl6 mice as well as fibroblasts from AKR mice and C57Bl mice were used. Three hundred cells suspended in 10 μl of medium were plated into each well of the microtest plates (Falcon Plastics). The cells were incubated for 6 hr in a gassed incubator in order to let the target cells adhere to the bottom of the wells. Prior to use the cells were washed to remove free cells and debris. The plating efficiency was between 100 and 200 cells per well. Three hundred, 3000 or 30,000 lymphocytes suspended in 20 μl of culture medium and in controls (20 μl of culture medium without cells) were added to the wells of each row of the microplates. In some experiments 10^7 lymphocytes were incubated with 0·2 ml of liver

homogenate (corresponding to 10 mg of protein) for 2 hr at 37°C; the preincubated lymphocytes were washed with culture medium prior to use in the microcytotoxicity test.

The plates were incubated in the incubator for 48 hr. After that time the test was terminated by washing the plates twice with cold medium to remove free target cells and the lymphocytes. The plates were stained with crystal violet and the intact cells were counted under an inverted microscope. The percentage of reduction of target cells was calculated according to the formula: percentage reduction = {[(number of cells in target cell control) − (number of cells in the test)]/(number of cells in target cell control)} × 100.

Statistics

Six to eight microcytotoxicity experiments, each carried out five times, were performed with each group. The results are given in Table 1. All statistical evaluations for significance were performed according to Student's *t*-test.

RESULTS

Histological examination of the livers showed that 73% of AKR mice immunized by repeated injections of allogeneic liver, developed a hepatitis which is characterized by intensive intralobular and portal infiltration with mononuclear cells as well as necrosis of liver parenchyma cells. Twenty-nine per cent of C57Bl6 mice immunized to AKR liver showed comparable liver damage. Apart from slight focal infiltration of the liver with mononuclear cells no liver damage was observed in animals immunized with syngeneic liver homogenate.

The results of the microcytotoxicity test using lymphocytes from *in vivo* immunized animals as effector cells are indicated in Table 1. Lymphocytes from AKR as well as C57Bl6 mice immunized to allogeneic liver *in vivo* showed a high cytotoxic activity to isogeneic and allogeneic liver target cells whereas control target cells as isogeneic fibroblasts or NCTC cells (established liver cell line of allogeneic origin) were not destroyed by the immunized lymphocytes. Limited cytotoxicity of lymphocytes was induced by immunization of mice to isogeneic liver cell antigen. The highest cytotoxicity was observed in the allogeneic system, i.e. when lymphocytes from C57Bl6 mice immunized to AKR liver were incubated with AKR liver cells or *vice versa*. In this allogeneic effector cell–target cell system cytotoxicity to the allogeneic fibroblasts, but not to the NCTC cells was demonstrated.

The cytotoxic activity of *in vivo* immunized lymphocytes which have passed through a nylon wool column was enhanced when compared with the unfractionated cells (Table 2). Non-sensitized cells did not kill after column passage. The passed cells were identified as mainly T cells. Treatment with anti-θ serum and complement killed 89% of passed lymphocytes from C57Bl mice; hence the passed cells represent an enriched T-lymphocyte population, but there was some contamination with non-T cells. Non-T cells from C57Bl mice prepared by treatment with anti-θ serum and complement were less cytotoxic on liver target cells than the unfractionated lymphocytes. The cytotoxicity, however, was definitely above that of the controls. The reduction of cytotoxicity after anti-θ serum treatment was a specific effect due to elimination of T cells. Incubation of unfractionated C57Bl lymphocytes with normal AKR serum instead of anti-θ serum did not inhibit killing. The reduction of target cells produced by lymphocytes after normal serum treatment was 25·6% at an effector cell : target cell ratio of 300 : 1.

TABLE 1. Cytotoxicity of lymphocytes from AKR mice or C57Bl mice immunized to isogeneic or allogeneic liver

Lymphocytes from:	Immunization to:	N*	Percentage reduction of target cells§									
			AKR liver cells		C57Bl liver cells		AKR fibroblasts		C57Bl fibroblasts		NCTC cells	
			30†	300†	30†	300†	30†	300†	30†	300†	30†	300†
AKR	C57Bl liver *in vivo*	8	26·8‡	34·5‡	32·8‡	51·6‡	3·2	2·4	7·3	19·4‡	−9·3	4·0
AKR	AKR liver *in vivo*	6	7·6	18·4‡	5·2	12·8‡	−4·1	3·1	−2·4	1·9	2·4	6·3
AKR	—	4	1·8	4·3	−2·4	1·9	−3·8	1·8	−3·8	2·3	−4·6	1·0
C57Bl	AKR liver *in vivo*	6	34·6‡	46·3‡	12·9	27·3‡	18·3‡	26·9‡	2·0	3·2	4·1	7·4
C57Bl	C57Bl liver *in vivo*	6	3·3	4·8	9·4	20·1‡	−3·2	1·9	1·6	5·3	−6·2	5·8
C57Bl	—	4	4·2	5·4	−1·4	3·8	−4·8	2·5	−1·2	2·8	−4·0	6·8
AKR	C57Bl liver *in vitro*	6	22·3‡	45·6‡	28·4‡	48·6‡	1·4	1·7	2·4	7·9	−14·1	−3·4
AKR	AKR liver *in vitro*	6	9·3	10·3‡	5·2	17·9‡	−1·2	4·1	−2·8	3·2	−14·2	1·2
AKR	No cells *in vitro*	6	−4·1	3·1	−4·3	1·9	−2·1	−1·0	−2·4	3·1	−9·2	1·4
C57Bl	AKR liver *in vitro*	6	23·8‡	48·3‡	4·9	31·6‡	2·7	8·6	−4·2	1·9	3·2	5·3
C57Bl	C57Bl liver *in vitro*	6	3·3	14·0	6·5	24·3‡	1·6	6·8	−3·1	1·8	2·1	4·9
C57Bl	No cells *in vitro*	6	1·5	3·2	−2·1	2·1	1·5	4·1	−4·8	−2·0	−6·1	3·7

* Number of cytotoxic experiments, each done five times.

† Effector cell: target cell ratio of 30: 1 or 300: 1 in the microcytotoxicity test.

‡ Values are significantly different from the control values at a level of $P < 0{\cdot}025$.

§ The geometric mean of the percentage reduction of target cells are given.

TABLE 2. Cytotoxicity of T cells and non-T cells from mice immunized to allogeneic liver on isogeneic liver target cells

Lymphocytes from:	Immunization to:	Target cells	Percentage reduction of target cells*					
			Unfractionated cells†		T cells‡		Non-T cells§	
			30¶	300¶	30¶	300¶	30¶	300¶
AKR	C57Bl liver	AKR liver	26·8	34·5	31·9	46·3	7·8**	12·3**
AKR	—	AKR liver	1·8	4·3	2·3	5·7	1·0**	2·9**
C57Bl	AKR liver	C57Bl liver	12·9	27·3	15·9	36·5	6·4	14·1
C57Bl	—	C57Bl liver	1·2	5·4	2·8	5·1	−0·5	1·8

* Geometric mean of the percentage reduction of target cells of three experiments, each done six times.
† Unfractionated cells = lymph nodes and spleen cells from immunized mice or non-immunized mice.
‡ T cells = lymph node and spleen cells from mice after passage through nylon wool column.
§ Non-T cells = lymph node and spleen cells from mice treated with anti-D serum and complement.
¶ Effector cell:target cell ratio of 30 :1or 300:1 in the microcytotoxicity test.
** Cells from AKR mice retained on nylon wool column and recovered by mechanical shaking.

Preincubation with isogeneic as well as allogeneic liver antigen reduced the cytotoxicity of lymphocytes to liver target cells significantly (Table 3). The cytotoxic activity was not impaired after incubation with isogeneic kidney antigen, whereas it was inhibited when the lymphocytes were incubated with allogeneic kidney antigen.

TABLE 3. Inhibition of lymphocytotoxicity after preincubation of lymphocytes from immunized mice with liver and kidney antigen

Preincubation of lymphocytes with	Percentage reduction of target cells*			
	AKR lymphocytes immunized to C57Bl liver		C57Bl lymphocytes immunized to AKR liver	
	30†	300†	30†	300†
—	26·8	34·5	12·9	27·3
Isogeneic liver	14·9	18·2	3·8	9·4
Allogeneic liver	2·3	9·8	1·3	4·8
Isogeneic kidney	24·3	36·2	9·6	21·4

* Geometric mean of the percentage reduction of target cells of three experiments, each done six times.

† Effector cell : target cell ratio of 30 : 1 or 300 : 1 in the microcytotoxicity test.

In vitro immunization of lymphocytes to liver cells led to similar results as the *in vivo* sensitization (Table 1). AKR lymphocytes immunized to C57Bl6 liver or C57Bl6 lymphocytes immunized to AKR liver were cytotoxic to AKR liver cells or to C57Bl6 liver cells, respectively. The lymphocytotoxicity was rather low in isogeneic experiments, i.e. in cytotoxicity assays using lymphocytes immunized to isogeneic liver as effector cells, and isogeneic liver cells as target cells. The effect was specific; in the controls fibroblasts as well as NCTC cells were not destroyed by the *in vitro* immunized lymphocytes.

DISCUSSION

In vivo immunization to allogeneic liver homogenate induces an experimental hepatitis. The inflammatory reaction of the liver is more severe in AKR strain mice immunized to C57Bl6 liver than in C57Bl6 mice sensitized to AKR liver antigen. This finding suggests a probable genetic predisposition of the AKR strain to produce experimental hepatitis under the conditions of our immunization procedure. It was not possible to induce histologically demonstrable liver injury either by immunization of mice to fractionated liver proteins or by injection of a crude syngeneic liver cell homogenate. The observation is in agreement with earlier results that allogeneic or xenogeneic liver antigens are more potent antigens in inducing an experimental hepatitis than syngeneic or autologous liver antigen (Scheiffarth & Warnatz, 1967; Meyer zum Büschenfelde, Kössling & Miescher, 1972). Lymphocytes of animals immunized to allogeneic or xenogeneic liver antigen reveal cell-mediated immune reactions to syngeneic liver antigen *in vitro*.

In the present experiments the intensity of the liver damage was correlated with the lymphocytotoxicity to liver target cells *in vitro*. Severe liver injury in AKR mice immunized to allogeneic liver homogenate corresponded to high cytotoxicity of lymphocytes from these mice to syngeneic or allogeneic liver target cells. In C57Bl6 mice with moderate liver damage the lymphocytotoxicity was lower than in the AKR mice. The lymphocytotoxicity induced by immunization to liver antigen is specific for liver cells. Lymphocytes which are cytotoxic for syngeneic liver cells do not destroy syngeneic fibroblasts. Fibroblasts, however, are lysed when they are incubated with allogeneic lymphocytes immunized to liver cells of the same allogeneic strain. Hence it can be assumed that fibroblasts do not carry antigenic determinants present on liver cells which are responsible for the cell-mediated immune response. Established mouse liver cells were not destroyed by the immunized lymphocytes, but the results with these target cells may be due to the fact that NCTC cells are not suitable for lysis.

Cytotoxic effects of lymphocytes are primarily attributed to T-cell function (Cerottini, Nordin & Brunner 1971); Perlmann and Holm (1969), however, have described an antibody-dependent cytotoxicity which is a function of non-T cells. In our experiments purified T-cell preparations are more cytotoxic than unfractionated lymphocytes. This finding suggests that the cytotoxic reaction to liver target cells observed in our experiments is due mainly to T-cell activity. However, it cannot be excluded that non-T cell-dependent cytotoxicity is also involved, since T cell-depleted lymphocyte population showed a low cytotoxicity on liver target cells.

Immunization to allogeneic liver cells induces higher cytotoxicity than immunization to syngeneic liver cells. The induced cytotoxicity is directed to allogeneic as well as to syngeneic liver cells, indicating that organ-specific common antigens are exposed on liver cells of several mouse strains. Two separate effects may be involved in the killing of allogeneic target cells: (1) the anti-histocompatibility antigen response; (2) an anti-liver response which is responsible for the destruction of syngeneic liver cells.

Lymphocytotoxicity can be inhibited by antigen (Baldwin, Embleton & Price, 1973). In our experiments the cytotoxic reaction of lymphocytes was inhibited after preincubation of lymphocytes with liver antigen whereas preincubation with kidney antigen was ineffective. The results suggest that the effector lymphoctyes have reacted with free antigen and are thus prevented from involvement in the cytotoxic immune reaction to specific antigenic determinants of liver cells. The findings of the blocking experiments support the concept that an anti-histocompatibility antigen response and an anti-liver response are involved in the lymphocytotoxicity to allogeneic target cells. For the cell-mediated immune reaction to syngeneic liver target cells, organ-specific antigeneic determinants seem to be responsible.

Lymphocytes *in vitro* incubated with liver cells become cytotoxic to liver target cells. In the *in vitro* studies, too, immunization to allogeneic liver cells is more effective than immunization to syngeneic liver cells. Despite the fact that the results of the *in vivo* and *in vitro* immunization experiments resemble each other the findings cannot be compared without reservation. For *in vivo* immunization we have used a crude liver homogenate, whereas the *in vitro* immunization was done against whole liver cells. Incubation of lymphocytes with liver homogenate in the Mishell–Dutton system did not induce significant lymphocytotoxicity to liver cells. Therefore it may be that the immune reactions of the *in vivo* and the *in vitro* immunized lymphocytes are directed against different antigens exposed on liver cells or present in the homogenate.

The findings of our *in vivo* and *in vitro* experiments are related to the problems of the recognition of self and organ-specific antigens (Jerne, 1971). Cohen and Wekerle (1973) have found in their studies that the control of autosensitization depends on the inhibition of the recognition of self. The natural tolerance of T lymphocytes for self antigens was abolished when T lymphocytes were incubated *in vitro* with autochthonous cells in the absence of autologous serum. They concluded from their results that tolerance to self antigens was regulated by serum factors which act on the lymphocytes.

In our experiments self-reactive lymphocytes were also activated *in vitro* by incubation with allogeneic or syngeneic liver cells in the absence of serum. To this extent our results correspond to the data of Cohen and Wekerle. Long-time immunization *in vivo* to allogeneic liver cell homogenate, however, also induces self-reactive lymphocytes which are cytotoxic to syngeneic liver cells and are responsible for the development of experimental hepatitis. The immunization of allogeneic cells bearing organ-specific antigens seems to induce an immune response with cross-reactivity to syngeneic or autologous cells with the same organ-specific determinants. Since the *in vivo* immunization occurs in the presence of autologous serum it has to be concluded that other mechanisms exist which allow lymphocytes to escape from the natural tolerance to self antigens.

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