Induction of Cytotoxic T Lymphocytes Specific to the Molecule of the Class I Major Histocompatibility Complex for Subcutaneous Immunization in Pads

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T lymphocytes from immune lymph nodes, specific to the molecule of the class I major histocompatibility complex, were found to contain cytotoxic T lymphocyte precursors which mature to become effector cytotoxic T lymphocytes only in the presence of helper cells and L3T4$^+$, but not Lyt2$^+$ T helpers. The findings indicate that a subcutaneous injection of alloantigen of the class I major histocompatibility complex for immunization in the pads leads to the creation of the type of microenvironment of the lymph nodes which prevents activation of Lyt2$^+$ T helpers or leads to the activation of their functionally negligible part.

Key Words: *alloantigen-specific cytotoxic T lymphocytes; helper cells; T helpers; processing*

Initiation of the immune response of cytotoxic T lymphocytes (CTL) to alloantigens of the class I major histocompatibility complex (MHC), just as to soluble antigens, involves the interaction of a T cell with an antigen-presenting cell. Studies of the immune response to mutant molecules of the class I MHC *in vivo* in the skin graft rejection test [10,12,14] and *in vitro* in a mixed lymphocyte culture [12,13] have helped elucidate the phenotypic heterogeneity of the T-helper population activated upon such an interaction. We now know that induction of CTL against the class I MHC is a result of interaction with T-helper subpopulations recognizing the alloantigen in different ways. Lyt2$^+$ T helpers specific to class I MHC molecules and L3T4$^+$ T helpers reactive to class I MHC molecules, processed and presented on their own antigen-presenting cells in the context of the class II MHC, belong to such subpopulations. Hence, both L3T4$^+$ and Lyt2$^+$ T helpers function, at least partially, at the expense of interleukin-2 secretion.

However, the alloreactivity of a particular T-lymphocyte subpopulation *in vivo* depends on the route of antigen presentation and on the mode of antigen delivery in the body. For example, only intravenous sensitization of C57Bl/6 mice with splenocytes of H-2K^b mutant mice (bm1) leads to suppression of the *in vitro* response of Lyt2$^+$ T helpers (proliferation and production of interleukin-2), but not of Lyt2$^+$ CTL [11]. Processing and presentation of a soluble antigen (ovalbumin) in the context of the class I MHC are observed only for intravenous injection but not during immunization intraperitoneally or in the pads [3].

We studied specific features of induction of CTL generated *in vitro* from lymphocytes of regional lymph nodes sensitized with class I MHC alloantigen *in vivo* by subcutaneous immunization in the pads. The system included *in vivo* sensitization and a period of culturing needed for maturation of CTL precursors in effectors. Hence, a study of the *in vitro* phase of the CTL response in this combined system might yield valuable in-

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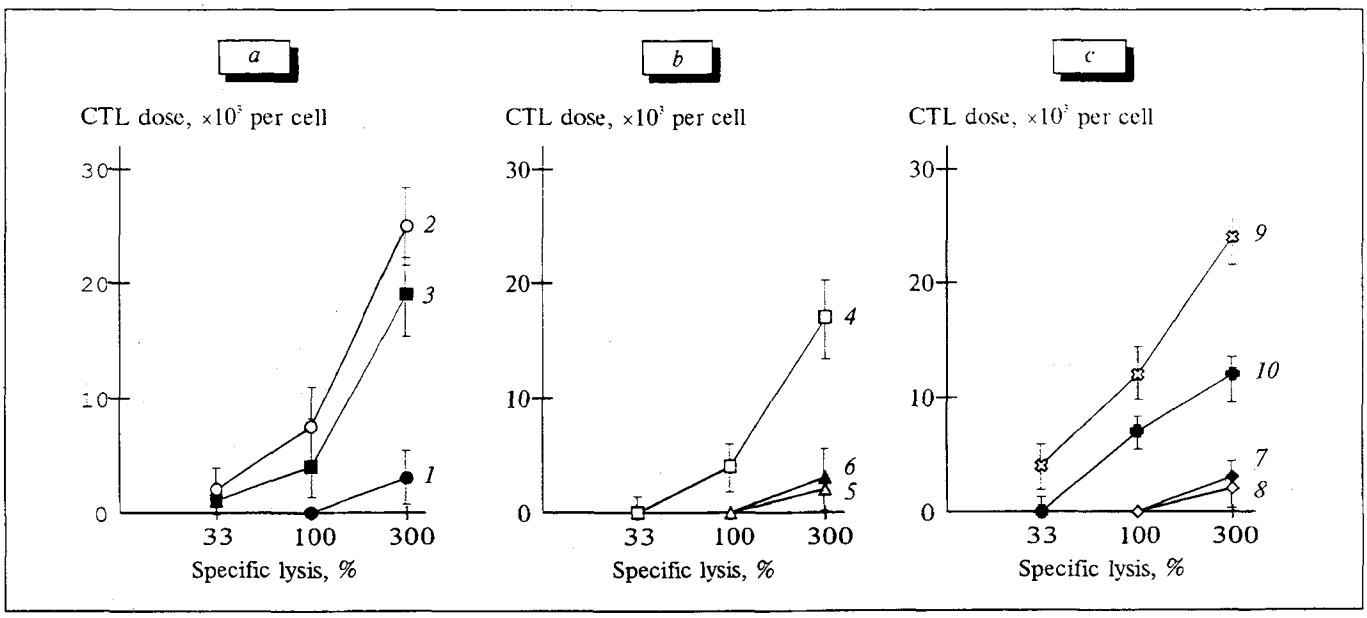

Fig. 1. Response of CTL *in vitro* generated from lymphocytes of lymph nodes sensitized *in vivo* with class I MHC alloantigen. CTL maturation *in vitro* in the absence of B lymphocytes (*a*), A cells (*b*), and L3T4⁺ T helpers (*c*). *1*) Total population of lymph nodes, newly isolated; *2*) total lymph node population after 3 days of culturing; *3*) T cells enriched by "panning" (removal of B cells); *4*) T cells enriched by "panning" and adhesion on petri dishes; *5*) T cells enriched by "panning", adhesion on petri dishes, and passing through Sephadex G−10; *6*) total lymph node population depleted of A cells by adhesion on petri dishes and passing through Sephadex G−10; *7*) total lymph node population devoid of L3T4⁺ T helpers; *8*) Lyt2⁺ T cells enriched by "panning", passing through Sephadex G−10, and cytolysis in the presence of antibodies to L3T4 antigen with the complement; *9*) Lyt2⁺ T cells cultured in the presence of supernatant from splenocytes stimulated with concanavalin A; *10*) Lyt2⁺ T cells cultured in the presence of interleukin−2 in a dose of 10 units/ml.

formation about the conditions of maturation of primary CTL specific to the class I MHC molecule, induced *in vivo*.

MATERIALS AND METHODS

C57Bl/6 (B6:$K^bI^bD^b$) mice and H-$2K^b$ mutant mice (B6.C-$H2^{bml}$) were bred at the breeding department of the Cancer Research Center, Russian Academy of Medical Sciences. For induction of primary CTL specific to the mutant molecule H-$2K^b$ a two-staged scheme previously described for minor alloantigens [4] was used with slight modifications. Recipients (C57Bl/6) were injected 10^7 irradiated (1500-2000 rad) splenocytes of donors (mice mutant for the H-$2K^b$ molecule, differing from mice of the initial strain in amino acid substitutions at sites 155 and 156) in serum-free medium in the front and hind pads. Four days after sensitization 5×10^6 cells of recipients' immune regional lymph nodes (popliteal, inguinal, subclavicular, axillary) were incubated for 3 days in 24-well plates (Flow) without antigen in RPMI-1640 medium with 10% fetal calf serum, 10 mM HEPES, 2 mM L-glutamine, 5×10^{-5} M 2-mercaptoethanol, and 100 U/ml gentamicin in a 5% CO_2 atmosphere at 37°C.

In some experiments the responder's lymph node T cells were fractionated. For fractionation different procedures were used individually or in succession: passages in petri culture dishes for 2 h at 37°C to remove helper cells adhering to plastic; passages in plastic petri dishes coated with antibodies to murine immunoglobulins (1.5 h at 4°C) to remove B cells; filtration through a column with Sephadex G-10 (Pharmacia) to remove helper cells [7]. When all three procedures were carried out in succession, the resultant population was very rich in T cells (98% of cells were lysed by monoclonal antibodies to Thy 1.2 antigen in the presence of complement). In other experiments L3T4⁺ and Lyt2⁺ T cells were separated. For this purpose T cells were treated for 40 min at 4°C with monoclonal antibodies to antigens of L3T4 (hybridoma RL 172.4) or Lyt2 (hybridoma 3.168) and then subjected to cytolysis in the presence of nontoxic rabbit complement at 37°C for 35 min. The cells were treated with antibodies with complement two times.

After culturing was over, the cells were washed and counted, and their cytotoxic index was assessed after 20 h incubation with ^{51}Cr-labeled target cells: donor and recipient macrophages (CTL response specificity control). The cytotoxic index was assessed by the formula: $(a-c)/(b-c)\times100\%$, where *a*, *b*, and *c* reflect, respectively, the experimental, maximal (in the presence of 1% Triton

X-100 solution), and spontaneous release of ^{51}Cr in the medium in cpm [6].

Supernatant of rat splenocytes stimulated with concanavalin A [1] and recombinant interleukin-2 (Biogen) were used as the source of exogenous lymphokines.

RESULTS

In vivo primed lymph node cells after 3-day culturing in a medium without antigen showed cytolytic activity towards target cells of the donor mouse strain. As was shown previously [2], a nonfractionated lymphocyte population of C57Bl/6 mice sensitized *in vivo* for 4 days with splenocytes of mice carrying the mutant class I MHC molecule weakly lysed allogenic targets directly after isolation from immune lymph nodes. However, after 3-day culturing the same lymphocytes developed appreciable cytolytic activity without the addition of exogenous lymphokines (Fig. 1, *a*). This indicates that in the system described the events which take place *in vitro* are critical for CTL generation. The absence of lysis on syngeneic targets helped assess the specificity of the CTL response.

For assessment of the mechanism of cell interactions leading to *in vitro* maturation of primary CTL, the cells of immune lymph nodes were fractionated. Bearing in mind that antigen-presenting cells of different types (macrophages, dendritic cells, B lymphocytes) direct the immune reponse to alloantigens, we removed them in succession before culturing.

Enrichment of the T-cell population by "panning" in petri dishes coated with rabbit antibodies to murine immunoglobulins (removal of B cells) did not lead to an increase of the functional activity of this population (Fig. 1, *a*). The role of B cells as alloantigen-presenters was demonstrated *in vivo* for the lymph nodes [9] and *in vitro* in a mixed lymphocyte culture [8]. However, their function as accessory cells (A cells) for induction of T helpers is not always realized because they are incapable of producing the necessary lymphokines, such as interleukin-1. The certain drop of the cytotoxic index after the removal of B cells (in comparison with the content of T cells in the nonfractionated population of the lymph nodes) might be due to the partial removal of accessory cells (macrophages and dendritic cells). Further enrichment of the T-cell population before culturing was carried out by adhesion on petri dishes for 2 h at 37°C and filtration through a column with Sephadex G-10. The results of this fractionation are presented in Fig. 1, *b*. The cells of immune lymph nodes devoid of A cells by this method but containing B lymphocytes also showed no cytotoxicity towards allogenic targets after 3 days of culturing (Fig. 1, *b*). Evidently, *in vitro* maturation of effector CTL completely depends on the presence of A cells, which may be effectively removed only by passing through Sephadex G-10 [7].

Virtually complete elimination of cytolytic activity was observed after culturing of the immune lymph node cells in the presence of $L3T4^+$ T cells (Fig. 1, *c*). Removal of $Lyt2^+$ T cells also abolished the CTL response (data not presented).

Hence, T lymphocytes obtained from immune lymph nodes evidently contain CTL precursors which mature to become effector CTL in the presence of A cells and $L3T4^+$ T helpers. The fact that the effect of A cells and $L3T4^+$ T helpers was replaced by supernatant from lymphocytes stimulated with concanavalin A containing the optimal set of lymphokines for the development of CTL (Fig. 1, *c*) is one more indirect piece of evidence. Recombinant interleukin-2 in a dose of 5 to 10 units/ml was less effective in this respect (Fig. 1, *c*).

Unfortunately, the genetic design of these experiments allows us to conclude only with certain reservations that the participation of A cells in the cascade of *in vitro* cell interactions leading to CTL maturation is realized via stimulation of $L3T4^+$ but not $Lyt2^+$ T helpers for secretion of interleukin-2 and other soluble factors. It is still not clear whether their *in vitro* interaction with $L3T4^+$ T helpers is restricted by the MHC complex. An alternative possibility is nonspecific stimulation of *in vivo* primed L3T4+ T helpers with growth factors produced by A cells.

Hence, the findings indicate that inoculation of class I MHC alloantigen by the aforesaid method results in the creation of such a microenvironment in the lymph nodes which prevents activation of $Lyt2^+$ T helpers or leads to the activation of their functionally negligible portion. One of the possible explanations of this effect is activation of the suppressor population *in vivo* in the lymph nodes, which selectively suppresses $Lyt2^+$ T helpers. Suppressor activity in the lymph nodes has been detected, for example, for immunization of mice with minor alloantigens [4,5]. On the other hand, an excess of antigenic stimulus in the limited space of the lymph nodes may lead to induction of tolerance at a clonal level. As a result, it is $Lyt2^+$ T helpers, which are characterized by the highest sensitivity to tolerization in comparison with $Lyt2^+$ CTL and $L3T4^+$ T helpers [11], that are no longer able to produce interleukin-2.

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Specific Features of the Effect of "Murine" Toxin and Antigen of *Yersinia pestis* Fraction I on Cells of Plague-Sensitive Animals

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Effects of *Yersinia pestis* capsular antigen and toxin on the cells of experimental animals are described. The antigen of bacterial fraction I enhanced the oxidative burst of white mice peritoneal leukocytes but suppressed the activity of guinea pig leukocytes. The effect of "murine" toxin was quite the contrary. Moreover, the effects of the toxin on the phosphorylation of leukocyte membrane and cytosol proteins of various origin differed, this correlating with fluctuations in the activity of tyrosine phosphatase.

Key Words: *plague; capsular antigen, "murine" toxin*

Yersinia pestis pFra plasmid with a molecular weight of 65 MD determines the synthesis of two products: "murine" toxin-exotoxin (MT) forming at 28°C and fraction I antigen (FIA) accumulating on the cell surface as a mucous layer at 37°C. Studies of the contribution of these substances to the development of plague infection have been carried out for a long time, but many aspects of the mechanism of their modulating effect on cells of the immune system of experimental animals (white mice and guinea pigs) are still not clear. MT (LD_{50} 0.6 to 1.25 μg) is known to be lethal for white mice, whereas guinea pigs are little sensitive to much higher doses. Conversely, FIA, a potent immunogen for white mice, rats, and monkeys, causes "immunoparalysis" in guinea pigs when used in the same doses [7,9].

In this research we compared the effects of FIA and MT on peritoneal leukocytes and macrophages of white mice and guinea pigs *in vitro*.

MATERIALS AND METHODS

Preparations of FIA capsular antigen isolated from *Y. pestis* [3] and toxin from *Escherichia coli* strain

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