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Modification of tumor cells by a low dose of Newcastle disease virus

II. Augmented tumor-specific T cell response as a result of CD4+ and CD8+ immune T Cell cooperation

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Summary. Augmented tumor-specific T cell responses were observed against the high metastatic murine lymphoma variant ESb when using as immunogen ESb tumor cells that had been modified by infection with a low dose of Newcastle disease virus (NDV). Such virus-modified inactivated tumor cells (ESb-NDV) were potent tumor vaccines when applied postoperatively for active specific immunotherapy of ESb metastases. We demonstrate here that immune spleen cells from mice immunized with ESb-NDV contain enhanced immune capacity in both the $CD4^+$, $CD8^-$ and the $CD4^-$, $CD8^+$ T cell compartments to mount a secondary-tumor-specific cytotoxic T cell response in comparison with immune cells from mice immunized with ESb. ESb-NDV immune $CD4^+$, $CD8^-$ helper T cells also produced more interleukin 2 after antigen stimulation than the corresponding ESb immune cells. There was no participation of either $CD4^+$ or $CD8^+$ virus-specific cells in the augmented response. The specificity of the T cells for the tumor-associated antigen remaind unchanged. Thus, there is the paradox that the virus-mediated augmentation of the tumor-specific T cell response in this system involves increased T helper activity but does not involve the recognition of viral epitopes as potential new helper determinants.

Introduction

In previous studies we have demonstrated that $CD4^+$ helper T cells were required (a) to establish resistance in DBA/2 mice against the syngeneic highly metastatic lymphoma ESb and (b) to develop an ESb-specific cytolytic T lymphocyte (CTL) response in vitro mediated by $CD8^+$ T cells [22]. Furthermore, it could be shown that viral modification of ESb tumor cells by a low dose of Newcastle disease virus (NDV) increases tumor immunogenicity in vivo [25] and the tumor-specific CTL response after sensitization in vivo and restimulation in vitro [24]. In a sensitive limiting-dilution, mixed leukocyte/tumor cell microculture system it was possible to demonstrate that viral modification led to a selective augmentation of the frequency of tumor-specific CTL clones. No CTL clones directed against viral antigens or new antigenic determinants were detectable [14].

The present study aimed at elucidating whether viral modification affected only $CD8^+$ cytotoxic T cells or also $CD4^+$ helper T cells. The experiments involve the use of CD4- or CD8-depleted immune spleen cells from mice immunized with ESb or ESb-NDV cells and analyzing the specificity and the capacities to produce IL-2 and to cooperate in the CTL response. The data will be discussed also in the context of the design of virus-modified tumor vaccines for immunotherapeutic application.

Materials and methods

Mice. DBA/2 mice 8–10 weeks of age were obtained from Charles River (Sulzfeld, FRG) and NMRI (nu/nu) mice from Bomholtgard (Denmark).

Antibodies and tumor cells. The rat monoclonal antibodies GK1.5 [6], RL172.4 [4] and 3.168.81 [20] were used after precipitation of ascites fluid from NMRI (nu/nu) mice with 40% ammonium sulfate. The DBA/2-mouse-derived tumor line ESb is a spontaneous, highly metastatic variant of the methylcholanthrene-induced T cell lymphoma L 5178YE (Eb). The tumor cell lines Eb and ESb were passaged in RPMI 1640 (Gibco) containing 10% FCS [13].

Immunization of mice. DBA/2 mice were immunized by injection into the external ear of either 5×10^4 ESb or ESb-NDV cells or 2×10^5 syngeneic spleen cells coupled with NDV (SSC-NDV). Four to eleven days later their spleen cells were tested for anti-tumor cytotoxic activity after restimulation in vitro.

In vitro induction of CTL. Nine days after priming the spleens were removed and $CD4^+$ or $CD8^+$ cells were eliminated by monoclonal antibody (mAb) and complement treatment as described below. Untreated, CD4- or CD8-depleted spleen cells (1.5×10^7) were restimulated with 1.5×10^6 inactivated ESb tumor cells (80 μg/ml mitomycin C, from Roth, Karlsruhe, FRG, for 1 h at 37° C) for 5 days in 7 ml complete culture medium. Tumor-specific cytotoxicity was then determined in a standard 4-h ^{51}Cr-release assay [13] against ESb and Eb tumor target cells.

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Abbreviations: CTL, cytolytic T lymphocytes; IL-2, interleukin 2; rIL-2, recombinant IL-2; mAb, monoclonal antibody; NDV, Newcastle disease virus; SSC, syngeneic spleen cell

For reconstitution of bulk cultures depleted of $CD4^+$ cells either 1.2×10^7 $CD8^-$ or 3×10^6 $CD8^+$ spleen cells were added to 1.2×10^7 $CD4^-$ spleen cells. $CD4^+$ spleen cells were prepared as follows: spleen cells of normal and immunized DBA/2 mice were enriched for T cells by passage over a nylon-wool column and $CD8^+$ cells were eliminated by treatment with mAb 3.168.81 and complement [22].

Elimination of $CD4^+$ and $CD8^+$ splenocytes. $CD4^+$ splenocytes were eliminated by sequential incubation with a mixture of mAbs GK1.5 (IgG_{2b}) and RL 172.4 (IgM) (ascites fluid diluted 1:100) and rabbit complement (Cedarlane Laboratories, Hornby, Canada, final dilution 1:20; 2 ml/10^8 cells). For elimination of $CD8^+$ cells, the mAb 3.168.81 (IgM) (ascites fluid diluted 1:100) was used. The efficiency of elimination was determined 4 days later by indirect immunofluorescence using the mAbs anti-CD4 (GK1.5), anti-CD8 (clone 53-6.7) [5] and anti-Thy1.2 (clone 30H12) [5] and, as second-stage reagent, a fluorescein-isothiocyanate-conjugated mouse anti-(rat κ chain) mAb. The number of $CD4^+$- or $CD8^+$-positive cells was reduced by more than 98% following the respective antibody treatment. The reduction of $Thy1.2^+$ cells corresponded to the proportion of the respective T cell subsets.

Virus propagation and infection. A stock of Newcastle disease virus of the avirulent strain Ulster was kindly provided by Dr P. J. Russel (Royal Veterinary College, London, UK) [10]. Virus was grown in the allantoic cavity of 10-day-old embryonated chick eggs. After 60 h at 36° C, allantoic fluid was collected and freed of debris by centrifugation at 800 *g* for 15 min. Further purification was achieved by centrifugation at 50000 *g* for 1 h. Virions were finally suspended in phosphate-buffered saline with 0.1% EDTA and stored at −70° C. For virus infection of stimulator or target cells 10^7 tumor or spleen cells were incubated for 1 h at 37° C with 160 HAU NDV and washed three times with phosphate-buffered saline.

Determination of IL-2 amount in $CD8^-$ bulk cultures. The amount of IL-2 in $CD8^-$ bulk culture supernatants was determined by measuring the proliferation of the IL-2-dependent growing T cell line w2 [18]. Surviving cell numbers were determined by the enzymatic cleavage of the tetrazolium salt MTT [3-(4,5-dimethylthiazol-2-yl)-2.5-diphenyltertazolium salt; Sigma no. M2928], which produces a dark-blue-coloured formazan derivative [17]. MTT cleavage was proportional to the cell number. In brief: 10^4 w2 cells were incubated with different dilutions of bulk culture supernatants in 96-well flat-bottom plates in a final volume of 200 µl/well. After 2 days 100 µl medium was removed and 20 µl MTT solution (5 mg/ml in phosphate-buffered saline) was added to each well. After 4 h 100 µl acidic isopropanol (40 ml 1 *N* HCL in 1000 ml isopropanol) was added to the wells and optical density was measured at 540 nm and 620 nm using a Flow MicroELISA reader. In the figures the difference between 540 nm and 620 nm was plotted [11]. The recombinant IL-2 (rIL-2) used as a standard was kindly provided by Sandoz, Vienna, Austria. It could be shown that the number of surviving w2 cells was proportional to the IL-2 concentration. The results were calculated as "lab units" according to the method of Gillis et al. [9], the linear part of the curves being used for linear regression analysis.

Results

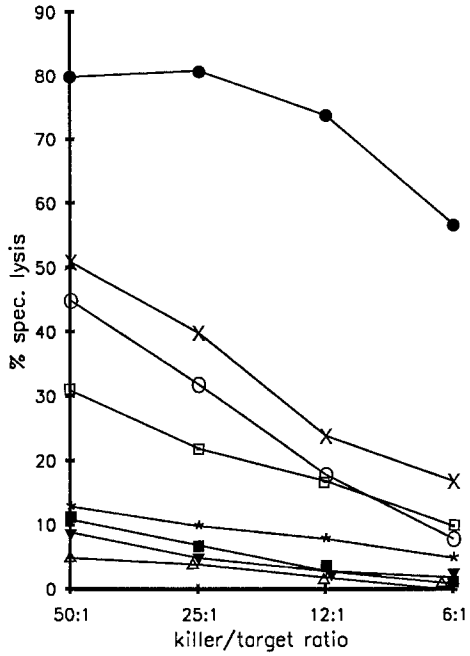
$CD4^+$ T cell requirement for augmentation of the CTL response to ESb-NVD

We showed in previous studies that modification of ESb tumor cells by infection with a low dose of NDV allows the production of a potent tumor vaccine, which can be used for postoperative immunotherapy of ESb micrometastases [12, 24]. The following experiments were performed to analyze the effects of viral modification on the tumor-specific immune response with regard to T helper cell involvement. Animals were immunized against either ESb-NDV or against ESb tumor cells alone. Nine days later the immune spleen cells were depleted of $CD4^+$ cells and tested together with unfractionated immune cells for their capacity to mount a secondary-tumor-specific cytotoxic T cell (CTL) response after in vitro restimulation with either ESb-NDV or ESb stimulator cells. The results are shown in Fig. 1. First it can be seen that the CTL activity of immune cells, sensitized and restimulated against ESb-NDV, was greatly augmented in comparison to that of immune cells sensitized and restimulated against ESb alone (cf. groups VII and I). Second, when $CD4^+$ cells were depleted by pretreatment with the mAb GK1.5 and complement, the responses against unmodified tumor cells (group II) as well as the augmented response against virus-modified tumor cells (group VIII) were abrogated. There was thus a strict $CD4^+$ T cell requirement both for the normal anti-tumor CTL response and for the increased response achieved by viral modification. The results in Fig. 2 also show that the cytolytic activity generated in the $CD4^+$-depleted cultures correlated inversely with the amount of antibody that was used together with complement to deplete the cultures. The higher the dilution of GK1.5 mAb, the more $CD4^+$ cells remained and the higher was the $CD8^+$ T-cell-mediated anti-tumor CTL response.

Comparison of $CD4^+$ and $CD8^+$ immune cells from ESb- or ESb-NDV-immunized mice in the cooperative CTL response

To investigate the function of $CD4^+$ and $CD8^+$ cells in the immune response against virus-modified ESb cells we performed the following experiments. Spleen cells of ESb- or ESb-NDV-immunized DBA/2 mice were depleted of either CD4- or CD8-positive cells, and combinations of such treated spleen cells were restimulated in vitro with ESb stimulator cells and assayed for CTL activity.

As can be seen from the control results displayed in Fig. 3, elimination of CD4- or CD8-positive cells totally abolished the in vitro CTL response of ESb- or ESb-NVD-immune cells. Tumor-specific CTL responses could be reconstituted, however, when CD4-depleted immune cells were coincubated with CD8-depleted immune cells in the presence of ESb stimulator cells. The extent of the CTL response was dependent on whether the cells were derived from ESb- or ESb-NDV-immune animals. A combination of CD4- and CD8-depleted spleen cells from ESb-NDV-immune animals showed the highest response, and those from ESb-immune mice, the lowest. An intermediate response was obtained from cocultures in which one T cell subpopulation was from ESb-immune and the other, from ESb-NDV-immune mice. These results thus clearly demonstrate that viral modification affected both $CD4^+$ and $CD8^+$ T cell subpopulations and that these subpopulations



Group

I	□	1° ESb, 2° ESb	V	X	1° ESb-NDV, 2° ESb
II	▼	I+GK1.5+C'	VI	△	V+GK1.5+C'
III	○	1° ESb, 2° ESb-NDV	VII	●	1° ESb-NDV, 2° ESb-NDV
IV	■	III+GK1,5+C'	VIII	*	VII+GK1,5+C'

Fig. 1. Effect of elimination of $CD4^+$ cells on the augmented CTL response after sensitization in vivo and/or restimulation in vitro with virus-modified ESb tumor cells. DBA/2 mice were sensitized against ESb (1° ESb) or ESb-NDV (1° ESb-NDV) cells by inoculation of 5×10^4 live cells into the external ear. Nine days later immune spleen cells were prepared and $CD4^+$ cells were eliminated as described. Normal splenocytes and splenocytes treated with mAb GK1.5 1:100 + complement (C') were restimulated with ESb (2° ESb) and ESb-NDV (2° ESb-NDV) cells for 5 days. The anti-tumor cytotoxicity was determined in a 4-h ^{51}Cr-release assay. The standard deviation of the arithmetic mean of three -eplicates was $<7\%$. This figure shows the lysis of ESb targets. Identical values were obtained for ESb-NDV cells. The lysis was ESb-tumor-specific since a similar tumor line with a different tumor antigen (Eb) was not lysed

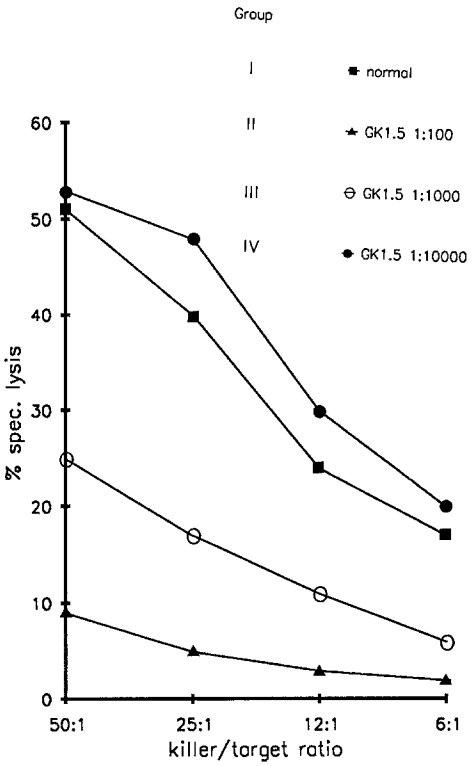


Fig. 2. Effect of elimination of $CD4^+$ cells on the tumor-specific secondary CTL response in vitro: dependence on the amount of anti-$CD4^+$ antibody. DBA/2 mice were immunized with 5×10^4 live ESb tumor cells intra pinna. After 9 days their spleens were removed and $CD4^+$ cells were eliminated with different dilutions of mAb GK1.5 in phosphate-buffered saline plus complement as described in *Materials and methods.* Untreated and GK1.5-treated splenocytes were restimulated with mitomycin-C-inactivated ESb cells for 5 days. Cytotoxicity was determined in a 4-h ^{51}Cr-release assay using the parental tumor line Eb, ESb and ESb-NDV cells as targets. Spontaneous ^{51}Cr release was $<10\%$. The values represent the arithmetic mean of three cultures with standard deviation $<10\%$. The figure shows the lysis of ESb cells. Identical values were obtained with ESb-NDV target cells, while unspecific lysis of Eb cells was $<5\%$

synergize, thereby giving rise to the augmented tumor-specific CTL response.

Comparison of $CD8^-$ immune cells from ESb- or ESb-NDV-immunized mice with regard to IL-2 production

Previous studies revealed that the defective CTL response in ESb-immune, CD4-depleted spleen cells could be reconstituted not only with $CD4^+$ cells but also with rIL-2 [22]. In order to compare the capacity of $CD4^+$ immune helper cells to produce IL-2 after antigen contact we tested in the following experiments $CD8^-$ spleen cells from ESb- or ESb-NDV-immunized mice for their ability to produce IL-2 after stimulation with ESb or ESb-NDV tumor cells.

Supernatants from bulk cultures of $CD8^-$ spleen cells of ESb- or ESb-NDV-immune mice, cocultured for 4 days with ESb or ESb-NDV, were tested for IL-2 content by adding them to the IL-2-dependent T cell line w2. After 24-h incubation the amount of w2 was determined by measuring the enzymatic cleavage of the tetrazolium salt MTT.

Figure 4a, b shows that immunization and restimulation with ESb-NDV (I) cells causes twice as much IL-2 production (6.5 units per culture) as immunization and restimulation with ESb cells alone (II) (3 units per culture). Besides the increased precursor CTL frequency [14], this increased IL-2 production may be a reason for the augmented CTL response.

No participation of virus-specific $CD4^+$ cells

The following experiments addressed the question as to whether the increased production of IL-2 and of CTL activity (Fig. 3) by ESb-NDV-immune cells was due to the participation of virus-specific $CD4^+$ T cells. Such cells could have been activated after in vivo immunization and in vitro restimulation with ESb-NDV cells.

To exclude differences in the number of $CD4^+$ cells in spleen cells after priming with normal or modified tumor

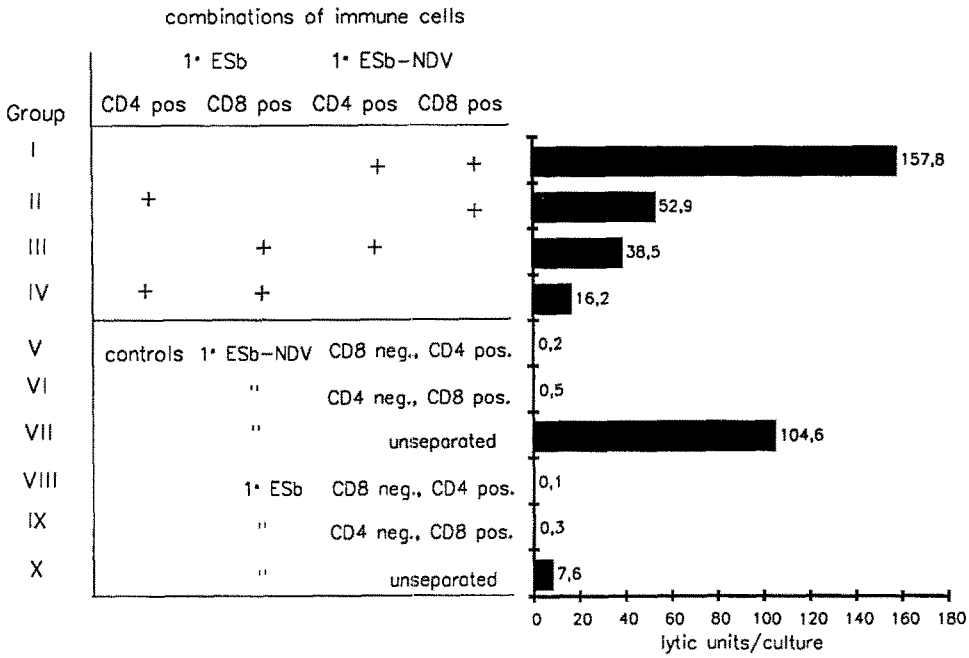


Fig. 3. Anti-tumor CTL activity of bulk cultures with ESb- or ESb-NDV-immune T cell subpopulations alone or in combination. DBA/2 mice were immunized with ESb or ESb-NDV cells intra pinna. After 9 days their spleens were removed and T cell subpopulations were eliminated. In the control cultures normal unfractionated, $CD4^-$ and $CD8^-$ splenocytes of ESb- and ESb-NDV-immune mice were restimulated as described. In the combination cultures 1.2×10^7 $CD8^-$ (CD4 pos) and 1.2×10^7 $CD4^-$ (CD8 pos) were mixed together and restimulated with inactivated ESb cells. Spontaneous ^{51}Cr release was $< 12\%$, and unspecific lysis of the parental tumor Eb was $< 4\%$. The values are expressed as lytic units/culture and were identical for ESb and ESb-NDV target cells. One lytic unit corresponds to the number of effector cells that cause 30% lysis of the target cells (5×10^3)

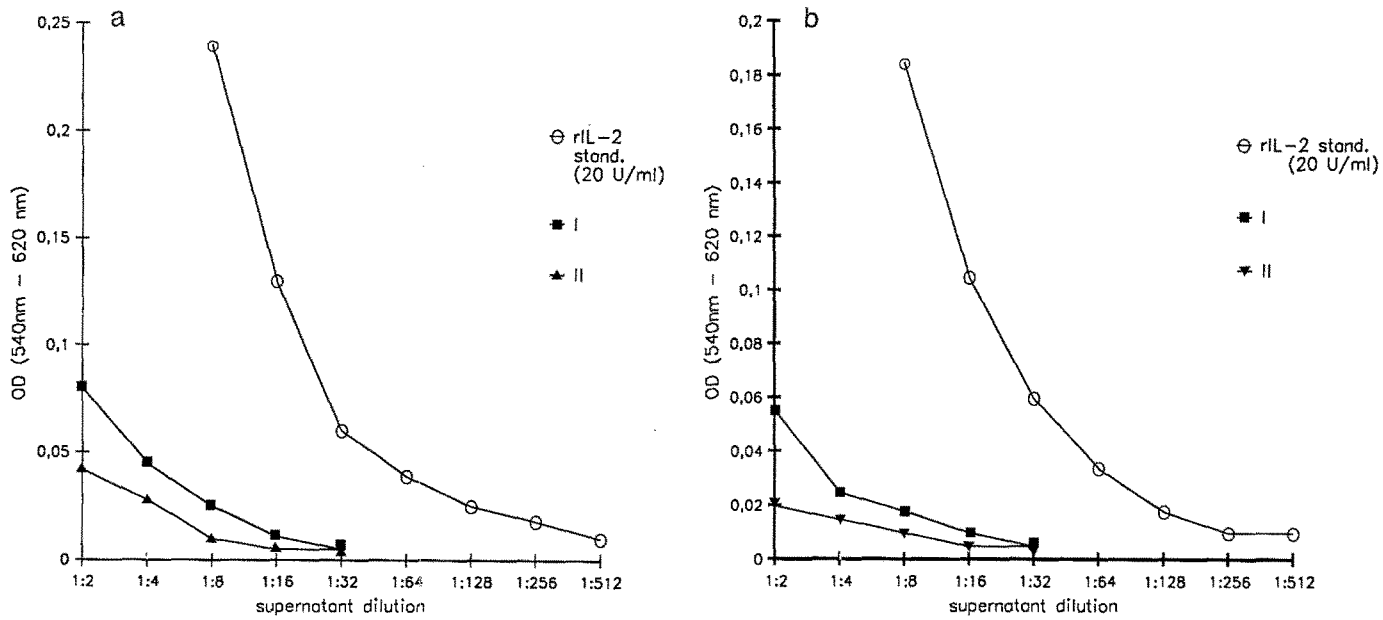


Fig. 4a, b. IL-2 production of $CD8^-$-immune splenocytes restimulated with ESb or ESb-NDV tumor cells. **a** DBA/2 mice were immunized with ESb cells and $CD8^-$ splenocytes were restimulated with ESb-NDV (I) or ESb (II) cells. After 4 days supernatants of these cultures were added to the IL-2-dependent growing T cell line w2. **b** DBA/2 mice were immunized with ESb-NDV (I) or ESb (II) cells and restimulated with ESb cells. IL-2 production was assayed as in **a**. Supernatants of bulk cultures not restimulated with ESb or ESb-NDV cells induced no proliferation of w2 cells.

cells, and to eliminate a possible influence of macrophages on the CTL response, $CD4^+$ cells were purified as described [22]. We added 3×10^6 $CD4^+$ spleen cells to 1.2×10^7 $CD4^-$ spleen cells and they were restimulated in a bulk culture. Table 1 contains the results of CTL activity in terms of lytic units per culture of cocultures of differently immunized CD4-positive and -negative spleen cells. $CD4^-$ spleen cells from ESb-immune mice alone showed nearly no CTL activity, regardless of what cells were used for restimulation (Table 1, group I). The response could be

Table 1. Reconstitution of the CTL activity of ESb-immune, $CD4^-$ splenocytes with $CD4^+$ immune cells from mice sensitized against ESb, ESb-NDV or syngeneic spleen cells plus NDV (SSC-NDV)[a]

Group	Immune cells	CTL activity (lytic units/culture) after restimulation with		
		ESb	ESb-NDV	ESb + SSC-NDV
I	ESb, $CD4^-$	0.8	0.1	0.7
II	ESb, $CD4^-$ + ESb, $CD4^+$	5.6	14.3	6.4
III	ESb, $CD4^-$ + ESb-NDV, $CD4^+$	10.6	23.8	9.6
IV	ESb, $CD4^-$ + SSC-NDV, $CD4^+$	0.3	1.7	1.2

[a] DBA/2 mice were immunized with ESb cells, their spleens removed after 9 days and $CD4^+$ cells eliminated. These cells (ESb, $CD4^-$) were reconstituted with $CD8^-$ ($CD4^+$) cells of differently immunized mice and the cocultures were restimulated with ESb, ESb-NDV and SSC-NDV. Reconstitution and preparation of $CD4^+$ cells is described in *Materials and methods*. Unspecific lysis of Eb cells was $<10\%$, spontaneous ^{51}Cr release from ESb cells was $<12\%$. The values show lytic units per culture with ESb cells as targets. Identical values were obtained for ESb-NDV target cells. Lytic activity was calculated as described in *Materials and methods* and Fig. 3

reconstituted by the addition of $CD4^+$ helper cells from ESb- or ESb-NDV-immune mice (groups II and III). $CD4^+$ cells from ESb-NDV-immune mice (group III) gave a higher response than $CD4^+$ cells from ESb-immune mice (group II) when restimulated with ESb cells.

Coculture with $CD4^+$ cells from mice immunized against NDV-spleen cells (SSC-NDV) induced no anti-ESb CTL response after restimulation with ESb or ESb-NDV cells (group IV). This suggests that the amount of NDV used to modify the ESb cells or the normal spleen cells (160 hemagglutination units/10^7 cells) does not induce virus-specific $CD4^+$ helper cells. Also, when SSC-NDV were used as stimulator cells together with ESb cells there was no virus-specific helper effect to be seen in any of the cocultures (groups II–IV, cf. ESb + SSC-NDV and ESb-NDV).

Nevertheless, when the same spleen cell combinations (groups II and III) were restimulated with ESb-NDV cells the CTL response was augmented in comparison to using ESb stimulator cells (cf. ESb-NDV and ESb). Identical values were obtained for ESb and ESb-NDV target cells. Thus, no virus specificity was detectable either at the target cell level or at the stimulator cell level; and at on the effector cell level, no virus-specific T helper cells or precursor CTL were detectable under these immunization conditions [14].

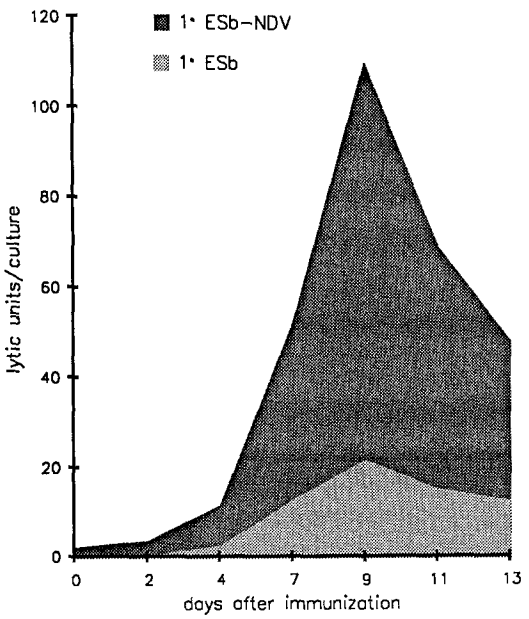


Fig. 5. CTL activity of restimulated DBA/2 splenocytes at different times following immunization against ESb or ESb-NDV cells. DBA/2 mice were immunized with 5×10^4 ESb or ESb-NDV cells intra pinna. After 0, 2, 4, 7, 9, 11 and 13 days their spleens were removed and the cells restimulated with inactivated ESb cells. Spontaneous ^{51}Cr release of ESb cells was $<13\%$ and unspecific lysis of the parental tumor Eb was $<4\%$ of maximal release. The figure shows lytic units per culture with ESb cells as targets. Identical values were obtained for ESb-NDV target cells

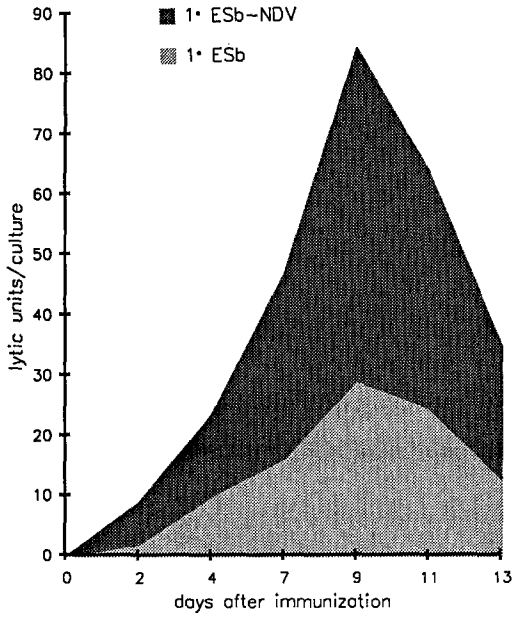


Fig. 6. Induction of CTL activity in $CD4^-$ splenocytes by $CD8^-$ spleen cells taken at different times after immunization with ESb or ESb-NDV cells. DBA/2 mice were immunized for 9 days with ESb cells and $CD4^+$ cells were eliminated from their spleen cells. These $CD4^-$ splenocytes were reconstituted with $CD8^-$ DBA/2 splenocytes immunized for 0, 2, 4, 7, 9, 11 and 13 days with ESb or ESb-NDV cells and restimulated with ESb stimulator cells. Spontaneous ^{51}Cr release of ESb cells was $<12\%$ and unspecific lysis of the parental Eb tumor was $<6\%$ maximal release. The figure shows lytic units per culture with ESb cells as targets. Identical values were obtained for ESb-NDV target cells

In spite of the absence of virus-specific $CD4^+$ cells, ESb-NDV-immune $CD4^+$ cells contained more helper activity than ESb-immune $CD4^+$ cells (cf. groups II and III, ESb), and ESb-NDV stimulator cells were more immunogenic than ESb cells (cf. ESb-NDV with ESb of groups II and III).

Time course of $CD4^+$ and $CD8^+$ immune cell activation in vivo after immunization with ESb or ESb-NDV

Because of the different CTL activity at the peak of the response after immunization with ESb or ESb-NDV cells, we tried to analyze the time course of $CD4^+$ T helper cell and $CD8^+$ precursor CTL activation. Figure 5 shows the overall CTL activity of ESb-restimulated bulk cultures of immune DBA/2 spleen cells at different times after in vivo immunization with ESb or ESb-NDV cells. The maximum of the CTL response in both cases was seen with spleen cells from day 9, but the extent of the response of ESb-NDV-immune cultures was increased threefold in comparison with ESb-immune cells. The ESb-NDV-immune response was higher over the whole time course, and a comparison of the two areas of activity revealed a threefold higher total CTL activity.

Similar results were obtained when we investigated the time course of $CD4^+$ immune helper cell activation. The activation of $CD4^+$ cells was determined as CTL activity obtained from bulk cultures where $CD4^-$ spleen cells from 9-day ESb-immune cells were combined with $CD4^+$ spleen cells from mice taken at different times after immunization with either ESb or ESb-NDV cells (Fig. 6). When mice were immunized with ESb-NDV cells the immune helper cell activity was higher and could be detected earlier than after immunization with ESb cells alone.

Discussion

In a previous report we documented that $CD4^+$ cells were required for induction of tumor resistance following intra pinna inoculation of 5×10^4 live ESb tumor cells in syngeneic mice. $CD4^+$ immune cells were also necessary to enable ESb-immune spleen cells to mount a secondary-tumor-specific cytotoxic T cell response in vitro [22]. The CTL response could be augmented by using NDV-modified tumor cells as immunogen [25]. Here we have demonstrated that the augmented CTL response is strictly dependent on the presence of immune $CD4^+$ T helper cells. Moreover, the $CD4^+$-mediated helper activity was itself affected by virus modification of the tumor cells. After immunization or restimulation with ESb-NDV the helper cells produce more IL-2 and induce a higher CTL activity than after immunization or restimulation with ESb cells. The specificity of the CTL response was not changed at all. $CD4^+$ cells, immunized with virus-modified syngeneic spleen cells, were not capable of inducing a CTL response when cocultured with ESb-NDV-immune $CD4^-$ spleen cells and restimulated with ESb or ESb-NDV cells. This showed that no virus-specific helper cells were involved. The CTL response and the "helper activity" could be augmented even when ESb-NDV cells were used only once, either for immunization in vivo or for restimulation in vitro. But ESb-NDV cells or syngeneic spleen cells coupled with NDV induced no CTL activity in vitro in primary mixed lymphocyte/tumor cell cultures. Immunization and restimulation with ESb and/or ESb-NDV cells was found necessary for induction and expression of tumor-specific immunity in both T cell compartments, the $CD4^+$ T-helper cell compartment and the $CD8^+$ CTL compartment. Since we could not detect any virus specificity we assume that the T-helper response of CD4-positive immune cells is tumor-specific just like the precursor CTL response of CD8-positive cells [14]. We showed before that ESb tumor cells express a distinct tumor-associated transplantation antigen, which induces protective immunity in vivo and tumor-specific CTL in vitro [2]. The recognition of the tumor-associated transplantation antigen on ESb target cells by CTL was restricted by the K^d class I MHC molecule [1]. The same or a different tumor-associated epitope may associate on antigen-presenting cells with a class II MHC molecule and be recognized by tumor immune T helper cells. This assumption is supported by the observation that anti-(class II MHC) mAbs could block the induction of a secondary CTL response in vitro [21]. Also, depletion of macrophages from tumor immune spleen cells abolished their ability to mount a secondary CTL response to ESb tumor cells in vitro [21].

Augmented tumor-specific T cell responses to virus-modified tumor cells have also been reported by others [1, 8, 26, 27], and Kobayashi has introduced the term viral xenogenization for this phenomenon [15, 16]. Since we have demonstrated in our system that there was no immunological recognition of foreign viral antigens either by $CD4^+$ T helper cells or by $CD8^+$ CTL immune cells, we think the term xenogenization may be misleading. We believe that the effect of modification of tumor cells by a low dose of NDV is more appropriately described as an effect of selective biological response modification of a tumor-specific immune response. The importance of virus dose has been emphasized before [12, 24]. The optimum of the therapeutic effect was seen with 160 hemagglutination units/10^7 ESb cells [12]. In order to induce a NDV-specific CTL response, animals had to be immunized for about 3 weeks with 8000 hemagglutination units of virus [14].

One explanation for the increased helper activity could be that viral hemagglutinins, which are sticky proteins, might facilitate the binding of adjacent tumor-associated transplantation antigens to the helper cell and thereby also activate $CD4^+$ cells with low-affinity receptors. A slight augmentation could be observed when ESb-NDV cells were used once, for immunization or restimulation. But these effects seem to synergize, so that the activation of helper cells appeared best when ESb-NDV cells were used both for sensitization and restimulation. Other important aspects to explain the augmented CD4-mediated "helper capacity" and CD8-mediated CTL activity of ESb-NDV-immune cells are the increased IL-2 production and the observed changes in the kinetics of the immune responses to ESb and ESb-NDV. When ESb-NDV cells were used as immunogen, T helper cells were activated faster and their response was higher in comparison to ESb as immunogen (Figs. 5, 6). The peak of both responses was at day 9. Thereafter, both responses declined but those from ESb-NDV-immune cells were always higher. When comparing the areas under the curves for lytic activity over, the total time period (Figs. 5 and 6), it can be seen that the ESb-NDV-immune response starts earlier, reaches a higher peak and lasts longer. The accelerated and augmented activation of tumor-specific CTL and T helper cells may be one reason for the effectiveness of ESb-NDV vaccines for antimeta-

static immunotherapy [12, 24-27]. We believe that strategies for cell-mediated immunotherapy of cancer should involve the augmentation of T–T cell interactions to increase tumor-specific delayed-type hypersensitivity responses [28] involving the recruitment and activation of host immunoreactivity [7, 10, 23, 24-29].

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