JOURNAL OF VIROLOGY, Oct. 1993, p. 6299–6302
0022-538X/93/106299-04$02.00/0

Vol. 67, No. 10

Divergence between Cytotoxic Effector Function and Tumor Necrosis Factor Alpha Production for Inflammatory CD4+ T Cells from Mice with Sendai Virus Pneumonia

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Received 29 March 1993/Accepted 9 July 1993

Sendai virus pneumonia in β2-microglobulin-deficient [β2-m(−/−)] mice lacking CD8+ T cells is characterized by the development of CD4+ cytotoxic T lymphocytes that can be recovered directly from the respiratory tract. These CD4+ cytotoxic T lymphocytes are not found in β2-m (+/+) mice, though inflammatory CD4+ T cells from both β2-m (−/−) and β2-m (+/+) mice produce substantial amounts of tumor necrosis factor alpha. Blocking experiments with a monoclonal antibody that also inhibits tumor necrosis factor beta show that the secreted forms of these two cytokines are not responsible for virus-specific killing of class II major histocompatibility complex-compatible targets. Comparison of electron micrographs indicates that the CD4+ effectors from the β2-m (−/−) mice are potent inducers of apoptosis, while this is not the case for the β2-m (+/+) CD4+ set. These experiments further define the functional status of virus-specific CD4+ T cells responding in vivo in the presence or absence of CD8+ effectors.

Mice that are homozygous (−/−) for a β2-microglobulin (β2-m) gene disruption express little class I major histocompatibility complex (MHC) glycoprotein and develop minimal numbers of CD8+ T lymphocytes (12, 25, 26). These β2-m (−/−) mice are able to survive infection (3, 8, 11, 23) with orthomyxoviruses (influenza A virus), a poxvirus (vaccinia virus), and a parainfluenza type 1 virus (Sendai virus). However, virus clearance is delayed and mortality is increased for the more virulent variants of these pathogens (3, 11). Also, the β2-m (−/−) mice are apparently unable to eliminate both the Theiler's murine picornavirus (9) and the arenavirus (13) murine lymphocytic choriomeningitis virus.

The mechanism of virus clearance by CD4+ effector T cells functioning in the absence of the CD8+ subset is not understood, though there is evidence from the influenza virus model that this mechanism may operate via CD4+ T-cell help for antibody-forming B lymphocytes (21). It is known that the small numbers of CD8+ T cells found in the β2-m (−/−) mice do not mediate virus elimination, at least in the Sendai virus model (11). Furthermore, CD4+ cytotoxic T lymphocytes (CTL) can be isolated directly from the spleens of β2-m (−/−) mice infected with lymphocytic choriomeningitis virus (7, 16) or in lymphocyte populations recovered by bronchoalveolar lavage (BAL) during the recovery phase of Sendai virus pneumonia (11). These CD4+ CTL are not present in β2-m (+/+) mice infected with viruses, though CTL effector function may be induced by in vitro culture.

Recent experiments (19) with vaccinia virus have shown that many of the responding CD4+ T cells in this infection produce the potent cytokine (24) tumor necrosis factor alpha (TNF-α). Also, immunosuppressed mice survived infection with a recombinant vaccinia virus expressing the gene encoding TNF-α, while those given the wild-type virus succumbed (19). We have thus investigated whether TNF-α is produced by the CD4+ T cells that localize to the lung of mice with Sendai virus pneumonia.

The capacity of freshly isolated CD4+ BAL T lymphocytes (1) from β2-m (−/−) mice to lyse Sendai virus-infected class II MHC+ LB15 (VLB) targets cells has been established previously by positive fluorescence-activated cell sorting (FACS) of the CD4+ subset and by blocking effector function with a monoclonal antibody (MAb) to the class II MHC glycoprotein (11). The characteristic CTL activity of such BAL populations for the ^{51}Cr-labeled VLB target cells and lack of lysis with the normal controls (NLB) are illustrated in Fig. 1. In this experiment, we also examined whether the lymphocytes in the BAL were, as a consequence of continued exposure to antigen expressed on unlabeled VLB cells, generating any diffusible factor that would mediate "bystander" lysis of allogeneic, uninfected

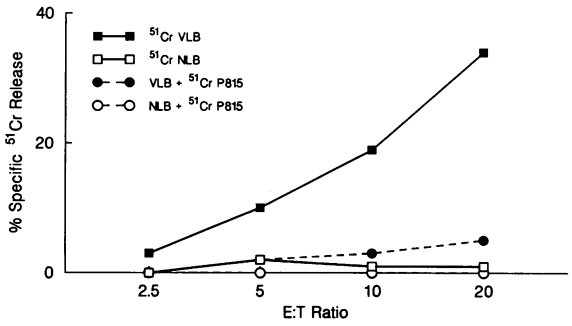

FIG. 1. Lymphocytes that do not adhere to plastic were recovered by BAL at 15 days after intranasal infection of β2-m (−/−) mice with 20 50% egg infective doses of the Enders strain of Sendai virus and exposed to various target cell populations in a 6-h ^{51}Cr release assay (11). The class I MHC+ class II MHC+, syngeneic (*H-2^b*) LB15 cells were (VLB) or were not (NLB) infected with virus (11). The allogeneic (*H-2^d*, class II MHC−) P815 cells were uninfected. The BAL effectors (E) were placed either with ^{51}Cr-labeled VLB or NLB targets (T) or with unlabeled VLB cells and ^{51}Cr-labeled P815 cells to assay for bystander killing. Individual wells of 96-well, U-bottom Falcon plates (Becton Dickinson Labware, Lincoln Park, N.J.) contained 5,000 target cells of each type in the mixed groups.

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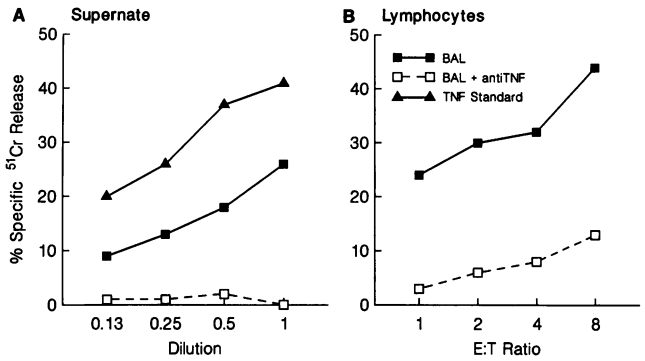

FIG. 2. The basic procedure and the source of the BAL lymphocytes were as described in the legend to Fig. 1. The lymphocytes were cultured together with 5,000 unlabeled VLB cells, which acted as targets or stimulators in the individual wells. Each microculture also contained 5,000 ^{51}Cr-labeled, uninfected, TNF-sensitive EL4 (*H-2^b*, class II MHC$^-$) target cells (10). The BAL supernatant consisted of pooled 1.0-ml samples taken from the first lung lavage (1) of different β2-m (−/−) mice. This material was concentrated fivefold in an Amicon B15 Macrosolute concentrator (Amicon, W. R. Grace & Co., Beverly, Mass.) and diluted as shown for incorporation in the assay. The undiluted TNF standard contained 6 U of recombinant TNF (Genentech, San Francisco, Calif.). The rabbit antibody to murine TNF (Genentech) was added to the assay wells to give a final dilution of 1:200. E:T, effector/target.

^{51}Cr-labeled P815 cells. No significant ^{51}Cr release was observed in such cultures (Fig. 1, VLB + ^{51}Cr P815).

The P815 targets that were used in Fig. 1 are not killed by TNF-α. We therefore repeated this bystander cytotoxicity assay by using a TNF-α-sensitive EL4 cell line that is class I MHC$^+$ class II MHC$^-$ and shares the *H-2^b* genotype of the β2-m (−/−) mice (10). In this case, the BAL lymphocytes caused substantial lysis of the ^{51}Cr-labeled, uninfected EL4 cells when incubated together with unlabeled VLB cells (Fig. 2B). Furthermore, lysis of the EL4 cells was greatly inhibited by incorporation of a rabbit antibody to TNF-α in the culture medium throughout the period of assay (Fig. 2B, BAL + antiTNF).

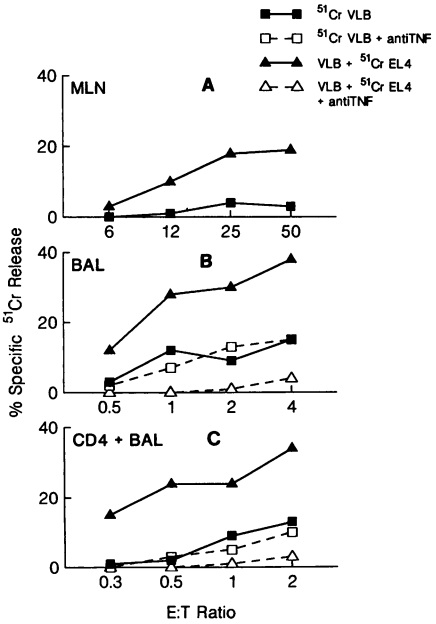

FIG. 3. The experiment was done with effectors from the BAL, unlabeled VLB cells, and ^{51}Cr-labeled EL4 cells as described in the legends to Fig. 1 and 2. Mediastinal lymph node (MLN) cells were also assayed; the lack of secretion of TNF-α by the VLB targets is illustrated by the level of lysis (3%) for the lowest MLN effector/target (E:T) ratio. The CD4$^+$ T cells were positively selected with phycoerythrin-conjugated H129.19 (Boehringer Mannheim, Indianapolis, Ind.) from freshly isolated, plastic-adhered BAL populations (1, 11), using the FACStar Plus (Becton Dickinson, Mountain View, Calif.).

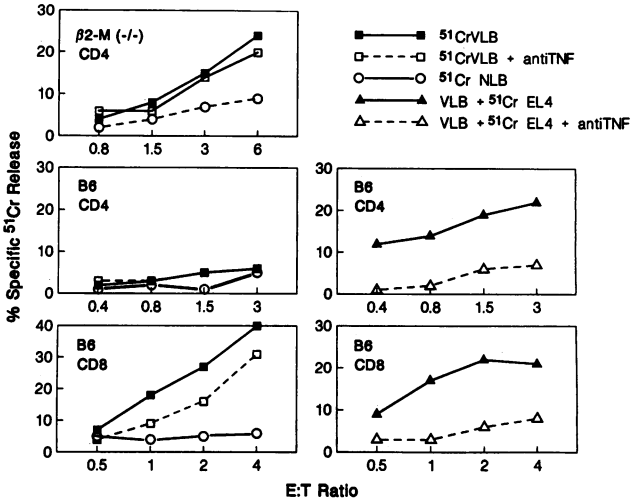

FIG. 4. Nonadherent (to plastic) BAL lymphocytes were recovered from B6 mice at 9 days after infection and from the β2-m −/− mice at 13 days after infection. The difference in timing is because the B6 mice clear the virus much more quickly (11). The CD4$^+$ and CD8$^+$ T cells were positively selected in two-color mode with phycoerythrin-conjugated H129.19 and fluorescein isothiocyanate-conjugated 53/6.7 (Pharmingen, San Diego, Calif.), using the FACStar Plus (1, 11). Both virus-specific CTL activity and TNF-α production were assayed as described above, with the exception that a neutralizing MAb to TNF-α (22) was used at a dilution of 1:400. E:T, effector/target.

Production of TNF-α in vivo during the course of this respiratory infection was shown by concentrating the BAL fluid phase and then using it to assay the EL4 targets. Substantial cytotoxicity, equivalent to that mediated by approximately 1.5 U of recombinant TNF-α, was found for these cell-free supernatants (Fig. 2A). All of the lytic activity was neutralized by the antibody to TNF-α (Fig. 2A, BAL + antiTNF).

Inflammatory cells other than CD4$^+$ T lymphocytes, particularly activated macrophages (10, 18, 24), are also likely to produce TNF-α. Most, but not all (1), of the macrophages are removed by the plastic adherence step used to enrich for the lymphocytes in the BAL (1) in these experiments (Fig. 1 to 3). Production of TNF-α by the CD4$^+$ subset was established by FACS separation from the nonadherent (to plastic) BAL population (Fig. 3C). All bystander lysis of the EL4 cells in the presence of the CD4$^+$ lymphocytes was again blocked by the polyclonal antibody to TNF-α, though CTL activity for the ^{51}Cr-labeled VLB targets was not inhibited (Fig. 3B and C). Similar results were recorded (Fig. 4) when a hamster MAb which neutralizes both TNF-α and TNF-β (20, 22) was used to inhibit cytotoxicity. Cells that produce TNF-α under these conditions of short-term in vitro culture (i.e., during the assay period) were also found to be present in the regional, mediastinal lymph nodes (Fig. 3A). How-

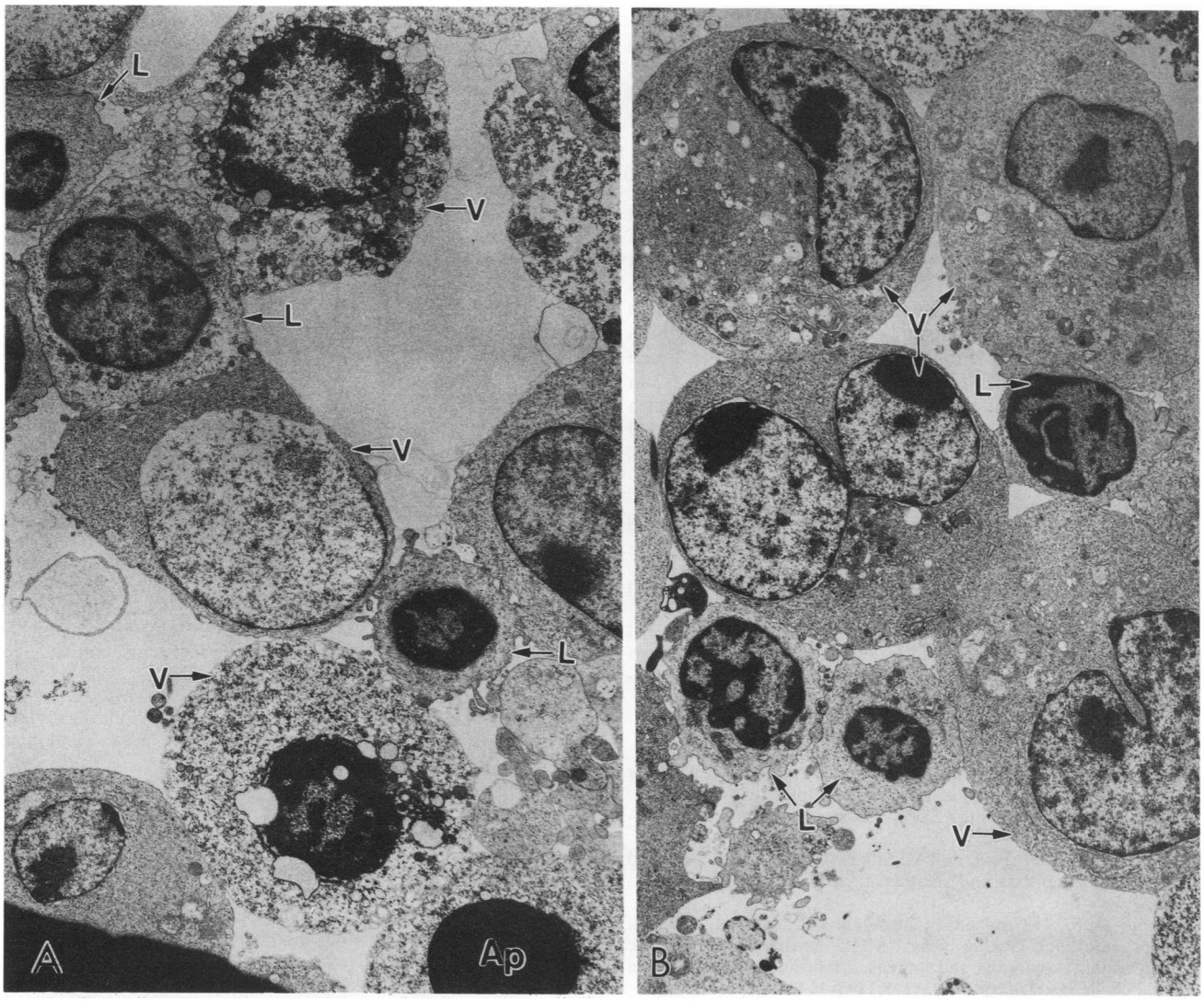

FIG. 5. The interaction between CD4$^+$ T cells recovered from the BAL of β2-m (−/−) (A) and β2-m (+/+) (B) mice and virus-infected LB15 cells is shown (magnification, ×3,000) for lymphocytes (L) and targets (V) that had been incubated together for 6 h at 37°C prior to fixation and embedding for electron microscopy (17). The virus-infected cells in contact with the lymphocytes from the β2-m (−/−) mice (A) are clearly undergoing apoptosis (6), as evidenced by convolution (blebbing) of the nuclear membrane, perinuclear accumulation of chromatin, and formation of dense apoptotic bodies (Ap). These changes are not seen for the targets associated with the β2-m (+/+) T cells (B). Virus-immune CD8$^+$ T cells from the β2-m (+/+) mice caused cytopathology similar to that seen in panel A (data not shown). The β2-m (+/+) lymphocytes were taken on day 9, and the β2-m (−/−) cells were taken on day 14, the difference reflecting the timing of peak CTL activity and the patterns of virus clearance in the two experimental systems (1). The BAL cells were adhered on plastic to remove macrophages, and the CD4$^+$ and CD8$^+$ sets were then isolated with the FACStar Plus in two-color mode (1).

ever, comparison of the effector-to-target ratios for the mediastinal lymph nodes and the BAL population (Fig. 3) indicates that they are much more prevalent in the virus-infected lung. Furthermore, both CD4$^+$ and CD8$^+$ T cells recovered from the lungs of β2-m (+/+) mice infected with Sendai virus produce TNF-α, though only the CD8$^+$ population contains virus-specific CTL effectors (Fig. 4).

We have thus demonstrated the presence of TNF-α in the respiratory tract of β2-m (−/−) mice that have been infected with Sendai virus and have established that this cytokine is being made by CD4$^+$ T lymphocytes. Sendai virus can cause the expression of TNF mRNA in cultured, human peripheral blood leukocytes (4), but there is no evidence that mouse T cells are infected with this virus in vivo, and all experiments were done at a time (day 15) when infectious virus had been cleared from the lung. Furthermore, CD4$^+$ T-cell-mediated cytotoxicity for class II MHC$^+$, virus-infected, syngeneic target cells is apparently not mediated by the secreted TNF-α or TNF-β. This finding does not, however, exclude the possibility that membrane-bound forms of either cytokine (5) may be involved. The underlying molecular events are yet to be definitively established for CD4$^+$ CTL in any experimental system, with a variety of possibilities currently being investigated (reviewed in reference 3).

The characteristics of the interaction between virus-infected targets and FACS-separated CD4$^+$ T cells recovered directly from the pneumonic lungs of β2-m (−/−) and β2-m (+/+) mice were further analyzed by electron microscopy

(17). The disruption of the nuclear membrane and margination of chromatin characteristic of apoptotic cell death (6) was prevalent for virus-infected targets in contact with the β2-m (−/−) $CD4^+$ T cells (Fig. 5A). This was not apparent for the $CD4^+$ β2-m (+/+) lymphocytes, despite their being intimately associated with the virus-infected LB15 cells (Fig. 5B). Thus, though TNF-α is produced by both lymphocyte populations, the activation states of inflammatory $CD4^+$ T cells recovered directly from the BAL of β2-m (−/−) and β2-m (+/+) mice are clearly different by the criteria of capacity to induce specific ^{51}Cr release and apoptosis (Fig. 4 and 5). Virus-specific $CD4^+$ T-cell clones derived from normal mice infected with an influenza A virus have also been shown to cause rapid disintegration of target cell nuclei (15). Persistent in vivo stimulation via the clonotypic T-cell receptor during the prolonged infection (11) in the β2-m (−/−) mice may simulate the effects of in vitro culture, with the virus-specific $CD4^+$ T cells being driven to a higher level of activation than is normally the case when $CD8^+$ effectors are also present in the site of pathology.

We thank Genentech for the gift of the recombinant TNF-α and the polyclonal antiserum, Robert Schreiber (Department of Pathology, Washington University, St. Louis, Mo.) for the hamster MAb to TNF-α, and Peggy Brown for assistance with the ultrastructural analysis.

This study was supported by grant AI-31596 from the National Institute of Allergy and Infectious Diseases, Cancer Center Support (CORE) grant CA-21765, and the American Lebanese Syrian Associated Charities.

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