Fine Specificity of Cytotoxic T Lymphocytes Primed In Vivo Either with Virus or Synthetic Lipopeptide Vaccine or Primed In Vitro with Peptide

By Hansjörg Schild, Maria Nörd, Karl Deres,* Kirsten Falk, Olaf Rötzschke, Karl-Heinz Wiesmüller,* Günther Jung,* and Hans-Georg Rammensee

From the Max-Planck-Institut für Biologie, Abteilung Immunogenetik; and the *Institut für Organische Chemie, Universität Tübingen, 72074 Tübingen, Germany

Summary

Standard synthetic peptide preparations contain numerous peptidic byproducts in small amounts, which may be efficiently recognized by cytotoxic T lymphocytes (CTL). Recognition patterns of such peptide mixtures by CTL may serve as a kind of fingerprint for CTL fine specificity. Three types of H-2Dd-restricted CTL were compared in this way. CTL primed in vivo either with A/PR/8/34 influenza virus or with a synthetic lipopeptide vaccine prepared from influenza nucleoprotein (NP) peptide 365-380 showed identical fine specificity. Both recognize virus-infected cells. In contrast, CTL primed in vitro with NP 365-380 had a different fine specificity and they did not recognize virus-infected cells. Most significantly, the two in vivo primed CTL types efficiently recognized the natural viral nonapeptide NP 366-374 presented by virus-infected H-2b cells, whereas the in vitro primed CTL failed to do so.

Materials and Methods

Virus Preparations and Mice. C57BL/6J (B6; H-2b) mice were bred and maintained at the animal facility of Max-Planck-Institut für Biologie. Strain A/PR/8/34 influenza virus was grown in the allantoic sacs of 11-d-old embryonated chicken eggs and stored as infectious allantoic fluid at −70°C. Infectivity of the preparation was tested by determining the titer of hemagglutinating units (HAU; reference 8).

Synthetic Peptides and HPLC. Peptides according to A/PR/8/34 influenza virus nucleoprotein amino acid residues 365-380 (NP 365-380; IASNENMETMESSTLE; reference 2) or NP 366-374 (ASNENMETM) and the lipopeptide P3CSS-NP 365-380 were synthesized and analyzed as described (3, 6, 7). Crude synthetic peptide preparations were separated by reversed phase HPLC using a SuperoxPep S column (Pharmacia LKB Biology Inc., Piscataway, NJ) as described (3). Individual fractions were collected, dried, dissolved in PBS, and used for CTL assays.

CTL Lines and Assays. All CTL lines were established by stimulating spleen cells with antigen for 5 d in 10 ml of α-MEM medium supplemented with 10% FCS, β-mercaptoethanol, L-glutamine, and antibiotics, followed by weekly restimulating CTL with irradiated (33 Gy) syngeneic spleen cells plus antigen in the above medium additionally containing Con A-induced spleen cell supernatant as a source of IL-2. For the line 19C90, a B6 mouse was immunized with 50 HAU of PR8 virus intravenously. 7 d later, 2 × 10⁷ recipient spleen cells were stimulated and restimulated in vitro with 1 gg/ml NP 365-380 peptide. The lines 29E90 and Hajo were derived from 4 x 10⁷ splenocytes of unprimed mice stimulated with 1 gg/ml of NP 365-380 peptide, fol-
lowed by weekly stimulation using the same peptide. CTL assays were performed as described (3, 7). E/T ratios ranged from 8:1 to 24:1; spontaneous release of target cells between 8.8 and 12.8%.

**Results**

**CTL Lines.** Three types of CTL lines are investigated in this study. The first type, exemplified by 19C90, is a virus-specific CTL line produced in the traditional way by priming a mouse (B6) with PR8 influenza virus, and stimulating splenocytes with virus in vitro. If prepared from H-2b mice, such CTL predominantly recognize an epitope contained in NP 365-380 (of influenza nucleoprotein) and are restricted to D\(^b\) (2). A variant of this first type is the CTL line 28890, also derived from a virus-primed mouse, but stimulated in vitro with NP 365-380 peptide. The second type of CTL (14C90) was derived from a mouse primed in vivo with a novel synthetic peptide vaccine (7), consisting of a lipotripeptide (P3CSS) covalently linked to NP 365-380. Splenocytes of this mouse were stimulated in vitro with NP 365-380 peptide. Both CTL types mentioned so far recognize virus-infected cells as well as target cells incubated with NP 365-380. The third type of CTL is produced entirely in vitro: spleen cells from unprimed mice were stimulated and restimulated with NP 365-380, as described (5). As exemplified by the lines 29E90 and Hajo, this type of CTL does not recognize virus-infected cells, although peptide-incubated target cells are readily recognized (see below), confirming earlier data (5). The line 29E90 has been tested to be D\(^b\) restricted and to be CD4-CD8\(^+\) (not shown).

**Reactivity of CTL with HPLC-separated NP 365-380 Preparation.** A standard synthetic peptide prepared according to the NP 365-380 sequence was separated by reversed phase HPLC (3). The main peptide elutes at the fraction corresponding to 41 ml elution volume. Some additional, rather small OD 220 peaks representing peptidic byproducts elute between 30 and 50 ml (Fig. 1, top). The arrow in Fig. 1 indicates the elution behavior of the nonapeptide ASNENMETM, which is the only one presented by virus-infected cells to common D\(^b\)-restricted, nucleoprotein-specific CTL (3, 9). Individual fractions were tested for recognition by CTL at three different dilutions of fractions. It is evident that all in vivo primed CTL (28890, 14C90, and 19C90) exert a rather similar recognition pattern (Fig. 1). All recognize most peptide fractions at high concentrations (at dilution 1:10). Many distinct peptides are still recognized at the following two dilutions.
Recog

nition of synthetic ASNENMETM again indicates identical sensitivity for the in vivo lines, whereas the in vitro primed line needs $10^2$-fold more of this peptide to reach the same level of lysis as the in vivo primed ones (Fig. 2 c). Thus, the relative failure to recognize the natural nucleoprotein peptide presented by $D_b$-expressing, PR8-infected cells appears to be the reason that CTL primed in vitro against free synthetic NP 365-380 peptide do not recognize virus infected target cells (5), as seen again in Fig. 2 d. On the other hand, a significant difference in fine specificity or sensitivity among the CTL primed in vivo with virus or with synthetic lipopeptide vaccine was not observed.

**Discussion**

Our data demonstrate that influenza nucleoprotein-specific CTL primed in vivo with virus or with a novel synthetic lipopeptide vaccine on the one hand and those primed in vitro with synthetic free peptide on the other hand differ in their fine specificity. Both types of in vivo primed CTL recognize virus-infected cells, whereas in vitro primed CTL fail to do so, confirming a previous report (5). Significantly, both types of in vivo primed CTL, whether tested as lines or as bulk cultures (3, 7), efficiently recognize the naturally processed peptide ASNENMETM produced by virus-infected $H_2$-K^d cells, whereas the in vitro primed CTL fail to do so; they need $10^2$-fold more of this nonapeptide for recognition, as compared with in vivo primed CTL. In contrast, both in vivo and in vitro primed CTL efficiently recognize a crude NP 365-380 peptide preparation, with only a small difference (10-fold) in efficiency. We conclude that the failure of in vitro primed CTL of the kind described here (and probably also in a previous report [5]) to recognize virus-infected cells is predominantly a consequence of their inability to efficiently recognize the naturally processed peptide presented by MHC molecules of virus-infected cells. In addition, a minor difference in the sensitivity to recognize peptide, or affinity of T cells (5), may also contribute to that failure.

The present data strengthen the notion that peptide purity is essential if any assay measuring T cell recognition is applied (3). Accordingly, we attempted to induce in vitro primed CTL by using synthetic free ASNENMETM peptide, corresponding to the natural $D_b$-restricted influenza peptide (M. Norda, unpublished results). These attempts failed, however, possibly due to self killing of CTL through recognition of free peptide (10).

Another practical aspect of our data is that normal synthetic peptide preparations are, in fact, complex mixtures of related peptides. Thus, recognition of different fractions of HPLC-separated synthetic peptides may serve as a kind of "fingerprint" for CTL fine specificity.

The different fine specificity of CTL primed in vitro with NP 365-380 peptide, as compared with the fine specificity of CTL primed in vivo with the corresponding lipopeptide containing the same sequence, appears to be a paradox. Since lipopeptides were ineffective for priming CTL in vitro (K. Deres, unpublished results) we are comparing here the effects

**Figure 2.** Titration experiments. Crude NP 365-380 peptide (a) or the fraction corresponding to 29-ml elution volume of HPLC-separated NP 365-380 (see Fig. 1 (b), or synthetic ASNENMETM (c) was tested in titrated concentrations for recognition by CTL lines 28B90 (●), 14C90 (▼), 29E90 (▲), or 19C90 (■). (D) Recognition of virus-infected EL4 cells by three of the above CTL.
of lipopeptides in vivo and free peptides in vitro. One possible explanation for this apparent paradox is that the 16-met peptide coupled to P3CSS, by virtue of P3CSS membrane affinity (11, 12), passes through cell membranes in vivo and joins the MHC class I-restricted processing pathway (13). As a consequence, the correct natural epitope can be cut out of the larger peptide and can be presented by MHC molecules. In contrast, synthetic peptides, even when mixed with P3CSS-OH (but not covalently coupled), may not enter cells, and therefore are inefficient for in vivo priming (13).

Similarly, a P3CSS-lipopeptide vaccine induces virus-neutralizing antibodies, whereas a mixture of P3CSS-OH and viral peptides is nonprotective (6). Synthetic peptides in vitro, on the other hand, may bind directly to cell surface class I molecules efficiently enough to be recognized by CTL. Since the processing devices of the stimulating cell are circumvented this way, the different fine specificity of the resulting CTL, as compared with in vivo primed CTL, may be explained by presentation of different peptides.

We thank J. Klein for support, S. Faath for technical assistance, and L. Yakes for typing the manuscript.

K. Deres is supported by Studienstiftung des Deutschen Volkes. This work was supported by Sonderforschungsbereich 120 and 323.

Address correspondence to Hans-Georg Rammensee, Abteilung Immungenetik, Max-Planck-Institut für Biologie, Corrensstr. 42, W-7400 Tübingen, Germany. Hansjörg Schild's present address is the Howard Hughes Medical Institute, Stanford University Medical School, Stanford, CA 84305.

Received for publication 20 June 1991 and in revised form 12 September 1991.

References