Brief Definitive Report

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 Norda, Karl Deres,* Kirsten Falk, Olaf Rötzschke, Karl-Heinz Wiesmüller,* Günther Jung,* and Hans-Georg Rammensee

*From the Max-Planck-Institut für Biologie, Abteilung Immungenetik; and the *Institut für Organische Chemie, Universität Tübingen, W-7400 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-2D^b-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-2^b cells, whereas the in vitro primed CTL failed to do so.

Virus-infected cells present viral nona- or octapeptides, bound to cell surface MHC class I molecules, to cytotoxic T cells (1–4). To induce such virus-specific CTL, in vivo priming is usually required. A recent report indicates that CTL against viral peptides can also be induced in vitro, and that such in vitro primed CTL are generally not able to lyse virus-infected target cells (5). This failure was ascribed to the low affinity of CTL primed in vitro as opposed to those primed in vivo. The peptide used (nucleoprotein [NP] 365-380) was longer than that presented naturally by virus-infected *H-2^b* cells, which is NP 366-374. Using the same long peptide (NP 365-380), coupled to a lipophilic anchor molecule containing three fatty acids (the lipotripeptide tripalmitoyl-*S*-glyceryl-cysteinyl-seryl-serine [P_3CSS] [6]), for immunization in vivo, we found that the resulting CTL were readily able to recognize influenza virus–infected cells (7). These CTL were as efficient as those primed with virus in vivo. The difference in the ability of CTL primed with virus or P_3CSS-NP 365-380 in vivo and those primed with NP 365-380 peptide in vitro prompted us to compare the fine specificity of the three different types of CTL by testing recognition of the numerous peptidic byproducts present in standard synthetic peptide preparations (3).

Materials and Methods

Virus Preparations and Mice. C57BL/6J (B6; *H-2^b*) 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 P_3CSS-NP 365-380 were synthesized and analyzed as described (3, 6, 7). Crude synthetic peptide preparations were separated by reversed phase HPLC using a SuperpacPep 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, 2×10^7 recipient spleen cells were stimulated in vitro with virus. Emerging CTL were stimulated weekly with virus. The line 28B90 was also derived from a mouse immunized in vivo with PR8 virus intravenously. The line 14C90 was derived from a mouse immunized in vivo with 100 μg P_3CSS-NP 365-380 lipopeptide intravenously. In both latter cases, recipient splenocytes were stimulated and restimulated in vitro with 1 μg/ml NP 365-380 peptide. The lines 29E90 and Hajo were derived from 4×10^7 splenocytes of unprimed mice stimulated with 1 μg/ml of NP 365-380 peptide, fol-

 J. Exp. Med. © The Rockefeller University Press • 0022-1007/91/12/1665/04 $2.00
Volume 174 December 1991 1665–1668

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-2*^{b} 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 28B90, 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 (P_3CSS) 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 (28B90, 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 di-

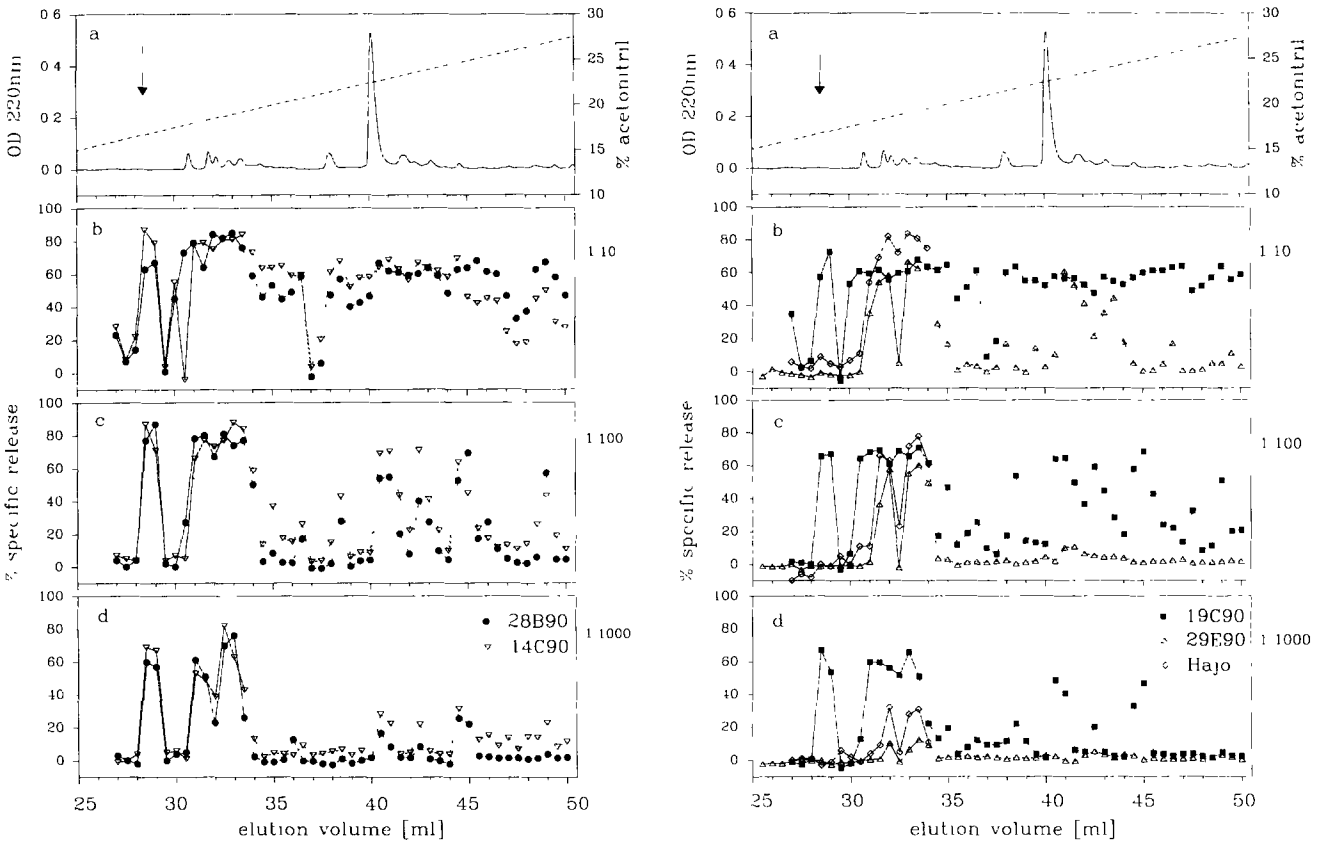

Figure 1. (*Left*) Fine specificity of CTL lines 28B90 and 14C90. (*a*) Synthetic NP 365-380 peptide was separated by reversed phase HPLC. (——) Optical density at 220 nm; (——) percent acetonitrile in the elution gradient. (*b–d*) recognition of individual HPLC fractions at 1:10 (*b*), 1:100 (*c*), and 1:1,000 (*d*) dilutions. Each fraction was incubated with EL4 target cells and subsequently tested for recognition by 28B90 CTL (●) or by 14C90 CTL (▽). (*Right*) Fine specificity of CTL lines 19C90, 29E90, and Hajo. (*a*) Separation of NP 365-380. (*b–d*) recognition of individual HPLC fractions at 1:10 (*b*), 1:100 (*c*), and 1:1,000 (*d*) dilutions. Each fraction was tested using 19C90 (■), 29E90 (△), or Hajo (◇) CTL.

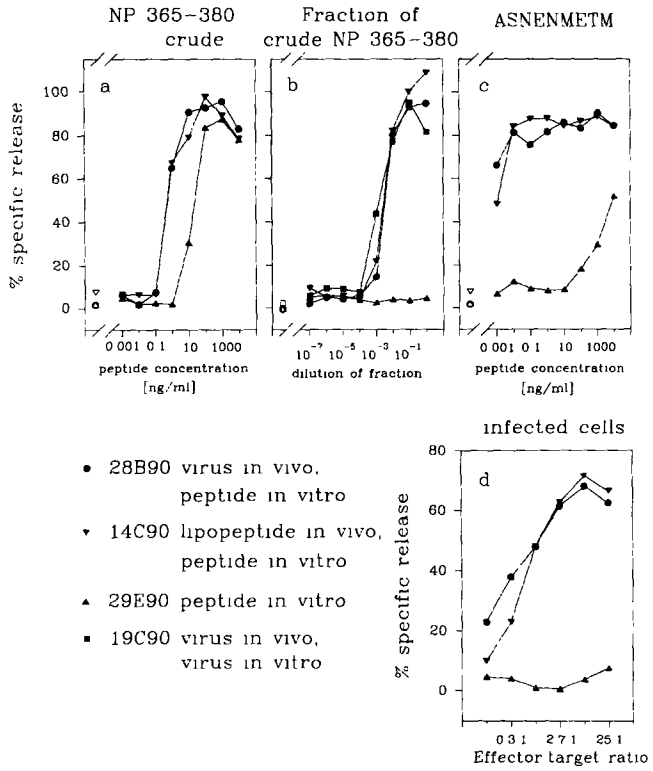

Figure 2. Titration experiments. Crude NP 365-380 peptide (*a*) or the fraction corresponding to 29-ml elution volume of HPLC-separated NP 365-380 (see Fig. 1 (*b*), or synthetic ASNENMETM (*c*) was tested in titrated concentrations for recognition by CTL lines 28B90 (●), 14C90 (▽), 29E90 (▲), or 19C90 (■). (*D*) Recognition of virus-infected EL4 cells by three of the above CTL.

lutions. Most notably, all three in vivo primed lines do recognize the fraction eluting at 28–29 ml with high efficiency, i.e., the fractions corresponding to the natural nonapeptide (3). The two in vitro primed CTL, 29E90 and Hajo, in contrast, show a reactivity pattern distinct from the former lines. Their main activity appears to be directed against the byproducts eluting between 30 and 35 ml. Even at the highest concentration, both lines do not recognize any material eluting at 28 or 29 ml. Thus, both in vitro primed lines appear not to recognize ASNENMETM, which is the peptide presented by virus-infected cells.

Titration Experiments. Titrated concentrations of a crude 16-mer peptide NP 365-380 preparation, of the fraction corresponding to 29 ml elution volume (corresponding to the natural peptide ASNENMETM) of HPLC-separated NP 365-380, and of purified synthetic NP 366-374 (ASNENMETM), were analyzed for recognition by the three CTL types (Fig. 2). Crude NP 365-380 is recognized by all of them. The in vivo primed lines 28B90 and 14C90 show no quantitative differences in their efficiency to recognize crude NP 365-380, whereas the in vitro primed line 29E90 needs ~10-fold more peptide to exert the same level of lysis as the in vivo primed lines (Fig. 2 *a*). All in vivo primed CTL show identical sensitivity for the fraction at 29 ml, whereas the in vitro primed line does not recognize this fraction at all (Fig. 2 *b*). Recognition of synthetic ASNENMETM again indicates identical sensitivity for the in vivo lines, whereas the in vitro primed line needs 10^7-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*^{b} cells, whereas the in vitro primed CTL fail to do so; they need 10^7-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 this highly efficient 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

of lipopeptides in vivo and free peptides in vitro. One possible explanation for this apparent paradox is that the 16-mer peptide coupled to P_3CSS, by virtue of P_3CSS 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 P_3CSS-OH (but not covalently coupled), may not enter cells, and therefore are inefficient for in vivo priming (13). Similarly, a P_3CSS-lipopeptide vaccine induces virus-neutralizing antibodies, whereas a mixture of P_3CSS-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, Correnssstr. 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

1. Zinkernagel, R.M., and P.C. Doherty. 1974. Restriction of in vitro T cell-mediated cytotoxicity in lymphocytic choriomeningitis within a syngeneic or semiallogeneic system. *Nature (Lond.).* 248:701.
2. Townsend, A.R., J. Rothbard, F.M. Gotch, G. Bahadur, D. Wraith, and A.J. McMichael. 1986. The epitopes of influenza nucleoprotein recognized by cytotoxic T lymphocytes can be defined with short synthetic peptides. *Cell.* 44:959.
3. Rötzscke, O., K. Falk, K. Deres, H. Schild, M. Norda, J. Metzger, G. Jung, and H.-G. Rammensee. 1990. Isolation and analysis of naturally processed viral peptides as recognized by cytotoxic T cells. *Nature (Lond.).* 348:252.
4. Van Bleek, G.M., and S.G. Nathenson. 1990. Isolation of an immunodominant viral peptide from the class I H-2K^b molecule. *Nature (Lond.).* 348:213.
5. Carbone, F.R., M.W. Moore, J.M. Sheil, and M.J. Bevan. 1988. Induction of cytotoxic T lymphocytes by primary in vitro stimulation with peptides. *J. Exp. Med.* 167:1767.
6. Wiesmüller, K.-H., G. Jung, and G. Hess. 1989. Novel low-molecular-weight synthetic vaccine against foot-and-mouth disease containing a potent B-cell and macrophage activator. *Vaccine.* 7:29.
7. Deres, K., H. Schild, K.-H. Wiesmüller, G. Jung, and H.-G. Rammensee. 1989. *In vivo* priming of virus-specific cytotoxic T lymphocytes with synthetic lipopeptide vaccine. *Nature (Lond.).* 342:561.
8. Zweerink, H.J., B.A. Askonas, D. Millican, S.A. Courtneidge, and J.J. Skehel. 1977. Cytotoxic T cells to type A influenza virus: viral hemagglutinin induces A-strain specificity while infected cells confer cross-reactive cytotoxicity. *Eur. J. Immunol.* 7:630.
9. Falk, K., O. Rötzschke, K. Deres, J. Metzger, G. Jung, and H.-G. Rammensee. 1991. Identification of naturally processed viral nonapeptides allows their quantification in infected cells and suggests an allele-specific T cell epitope forecast. *J. Exp. Med.* 174:425.
10. Walden, P., and H.N. Eisen. 1990. Cognate peptides induce self-destruction of CD8$^+$ cytolytic T lymphocytes. *Proc. Natl. Acad. Sci. USA.* 87:9015.
11. Wolf, B., S. Hauschild, B. Uhl, J. Metzger, G. Jung, and W. Bessler. 1989. Localization of the T cell activator lipopeptide in bone marrow-derived macrophages by electron energy loss spectrometry (EELS). *Immunol. Lett.* 22:121.
12. Praß, W., H. Ringsdorf, W.G. Bessler, K.-H. Wiesmüller, and G. Jung. 1987. Lipopeptides of the C-terminus of Escherichia coli lipoprotein: synthesis, mitogenicity and properties in monolayer experiments. *Biochim. Biophys. Acta.* 900:116.
13. Schild, H., K. Deres, K.-H. Wiesmüller, G. Jung, and H.-G. Rammensee. 1991. Efficiency of peptides and lipopeptides for in vivo priming of virus-specific cytotoxic T cells. *Eur. J. Immunol.* In press.