Cytotoxic T Cell Specificity for Respiratory Syncytial Virus Proteins: Fusion Protein Is an Important Target Antigen


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SUMMARY

We examined the specificity of BALB/c cytotoxic T (Tc) cells for respiratory syncytial virus (RSV) components, using recombinant vaccinia viruses (VV) coding for several individual RSV proteins. We found that immunization with the different VVs yielded the following Tc memory cell populations: high levels of RSV-specific Tc cells were induced with the fusion protein VV, but low levels were induced with VV coding for the RSV nucleoprotein. Tc cell recognition of attachment glycoprotein, part of the matrix molecule or IA internal protein was poor. While high levels of fusion protein-specific Tc cells were induced by the fusion protein VV, they showed poor cross-reactivity between the A2 and 8/60 RSV strains compared with Tc cells primed by RSV infection.

INTRODUCTION

Respiratory syncytial virus (RSV) is designated for vaccine development. It causes severe disease in infants and T cell responses need careful study. Cytotoxic T (Tc) cells have been described in mouse (Taylor et al., 1984b; Bangham et al., 1985) and in man (Bangham & McMichael, 1986). Murine Tc cells recognize target cells infected with antigenically distinct human RSV isolates (Bangham & Askonas, 1986) and their role in protection or immunopathology is being examined. So far, it has been shown that transfer of primed T cells can clear a persistent RSV infection in immunodeficient mice (Cannon et al., 1987). Whatever the role of Tc cells, we aimed to examine their specificity for RSV components for a rational design of vaccines.

In other infections, such as those caused by influenza virus (Bennink et al., 1984; Townsend et al., 1984; Yewdell et al., 1985), vesicular stomatitis virus (VSV) (Yewdell et al., 1986), reovirus (Finberg et al., 1982) and simian virus 40 (Gooding & O’Connell, 1983) Tc cells have been shown to recognize internal nucleoprotein and surface glycoprotein components, as well as non-structural components or polymerases (Bennink et al., 1982; Gotch et al., 1987). In the absence of natural RSV recombinant viruses differing in single proteins, or of easily purified viral components, one approach to the study of Tc cell specificities has employed vaccinia virus (VV) recombinants expressing individual viral genes. Thus, Bangham et al. (1986) have recently shown that Tc cells from mice infected with RSV and from humans after natural RSV infection, will recognize target cells infected with VV recombinants expressing the nucleoprotein (N), but will not detectably recognize those expressing the attachment glycoprotein (G).

In this study, we extended our previous study in the mouse by priming with a panel of VV recombinants expressing RSV fusion protein (F), G, matrix protein (M), non-structural protein (IA), or a higher level of N genes and showed that, of the VVs tested, only that expressing F would induce high levels of RSV-specific Tc cells, and that expressing N induced low levels. As the F-specific Tc cells in BALB/c mice appeared largely subtype-specific, this still left the major antigen(s) for cross-reactive Tc cells unidentified.

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Virus. RSV strains A2, 8/60 and 18537 (given by Dr E. J. Stott, AFRC Institute for Research on Animal Diseases, Compton, U.K.) were grown in HEp-2 cells (Bangham et al., 1985) and assayed for virus infectivity by a microplaque immunoperoxidase assay (Cannon, 1987). In brief, HEp-2 cells were grown in 96-well flat microtitre plates. Stock virus was diluted in 0.5 log, steps, and 50 μl aliquots were used to infect wells in duplicate for 2 h. After incubation overnight, cells were fixed and stained using monoclonal antibodies (kindly given by Dr B. Fernie, Georgetown University Schools of Medicine and Dentistry, Rockville, Md., U.S.A.) to viral antigens, using rabbit anti-mouse IgG antibody conjugated to peroxidase to form an insoluble product of 3-amino-9-ethylcarbazole. Each infected cell was counted as equivalent to 1 p.f.u. of virus.

Construction and selection of recombinant vaccinia viruses VAG 301 and VN 125 containing respectively the G (G VAC) or N (N VAC) gene cDNA of RSV A2 has been described (Ball et al., 1986). Additional recombinant VV vectors were prepared using essentially the protocol of Ball et al. (1986). VN 333 was a further recombinant containing the N gene cDNA, and it led to higher levels of N protein expression than VN 125 (tested by immunoprecipitation with rabbit anti-RSV sera; A. M. Q. King, S. Langer, L. A. Ball & G. W. Wertz, unpublished data). VF 325 (low expression) and VF 317 (higher expression of F) were two recombinants containing RSV F gene cDNA (F VAC) in the positive orientation (Wertz et al., 1987). VNG contained both N and G cDNA which expressed at high levels (NG VAC), VM contained 50% of the M cDNA (M VAC), and V1A4 contained cDNA coding for the IA protein (1A VAC).

Recombinant VV stocks were grown and titrated in HEp-2 cells as described (Bangham et al., 1986) and stored frozen at -70 °C; they ranged in titre from 2 × 10^9 to 3 × 10^10 p.f.u./ml.

Infection of mice. Female BALB/c mice aged 4 to 12 weeks and bred under specific pathogen-free conditions at this Institute, were infected intranasally (i.n.) with 2 × 10^5 p.f.u. (50 μl vol.) of RSV A2 strain. Mice receiving vaccinia recombinants were given either 5 × 10^5 p.f.u. i.n. (50 μl) or 2 × 10^7 p.f.u. intraperitoneally (i.p.) (100 μl).

Generation of Tc cells. Splenocyte cultures were stimulated with RSV A2 for 5 days as described (Bangham et al., 1985). Basic medium was RPMI 1640 plus 10% foetal calf serum containing penicillin (60 μg/ml), streptomycin (100 μg/ml), L-glutamine (300 μg/ml) and 2-mercaptoethanol (5 × 10^-5 M). Tc cell lines were generated by repeated restimulations in vitro every 7 days, with syngeneic A2-infected X-irradiated (3000 rad) spleen cells. Cultures were supplemented, after four or five restimulations, with 10% concanavalin A-pulsed rat cell supernatant as a source of interleukin 2. Tc cell line A2/P8 was derived from splenocytes primed with RSV A2 and restimulated with A2 in vitro eight times. Tc cell line F/P8 was derived from spleenocytes primed with VF325 and restimulated eight times in vitro with RSV A2.

Target cells and cytotoxicity assay. Target cells for Tc cells were P815 mastocytoma cells or BALB/c fibroblasts (continuous line), infected with RSV strains at a m.o.i. of 0·1 to 2 p.f.u./cell overnight. BCH4 cells are a BALB/c fibroblast line persistently infected with the Long strain of RSV (Fernie et al., 1981), and obtained from Dr E. J. Stott. BALB/c fibroblast lines were also persistently infected in this laboratory with RSV 18537. Uninfected P815 cells or BALB/c fibroblasts served as controls for non-specific lysis.

Tc cells were assayed using a 51Cr release assay as previously described (Bangham et al., 1985) using 10^4 target cells/well. The percentage specific lysis (L) was calculated as follows: 

\[ L = \frac{(\text{sample c.p.m.} - \text{background c.p.m.})}{(\text{total c.p.m.} - \text{background c.p.m.})} \times 100 \]

where total c.p.m. is the radioactivity released by targets treated with Triton X-100 detergent. Background release of 51Cr ranged from 10 to 30%.

METHODS

RESULTS

Viral components priming for Tc cells

The virus repertoire of Tc cells for RSV proteins could be tested by immunizing mice with recombinant VV coding for individual virus proteins and looking for priming of anti-RSV Tc cells. Fig. 1 illustrates results obtained following immunization with the different vaccinia vectors. Spleen cells of immunized mice were stimulated in vitro with A2 virus for 5 days and lysis of A2-infected target cells was examined. Quite clearly (Fig. 1a), F was a major target antigen for Tc cells and immunization i.p. primed for much higher levels of Tc memory cells in spleen than infection i.n. with VF 317. N VAC (VN 333) priming resulted only in low levels (10%) of RSV-specific target lysis (above background) and thus primed only low levels of Tc cells. G VAC (VAG 301) Tc cell priming was marginal, and M VAC or IA VAC did not induce significant levels of Tc memory cells (Fig. 1b). Fig. 1(a) illustrates a representative assay, but while the low level of Tc cell priming with N VAC was totally consistent, that induced by G VAC was not. The vaccinia vector containing genes for both N and G did not induce significant
levels of Tc memory cells either. The F VAC vector was not as efficient in priming Tc cells as intranasal RSV A2 infection of the mice. This could have been due to vaccinia priming or to induction of Tc cells with additional specificities.

**Tc cell cross-reactivity for different RSV strains**

We have previously shown that Tc cells primed by RSV A2 infection i.n. recognize target cells equally well whether infected with RSV A2 (group A) or RSV 8/60 (group B) (Bangham & Askonas, 1986). It was of interest to see whether the same would be true for F-specific Tc cells primed with F VAC. Fig. 2(b) shows that this was not so and that target cells infected with RSV 8/60 were barely better lysed than uninfected target cells, while Tc cells primed by infection i.n. with RSV A2 showed similar levels of lysis to the same batch of RSV A2- or RSV 8/60-infected targets (Fig. 2a). We did find that VV-infected mice showed higher non-virus-specific target cell lysis than RSV-infected mice.

Similarly, we tested Tc cell lines restimulated eight times in vitro with A2 virus following priming by A2 or F VAC infection: the Tc cell line from A2-primed mice lysed target cells infected with different RSV strains such as Long, 8/60 or A2 to a similar extent (Fig. 3a). In contrast, the Tc cell line from F VAC-primed mice showed low lysis of target cells infected with 8/60 virus compared to A2 and Long viruses (Fig. 3b). In this experiment we also tested lysis of a BALB/c fibroblast line persistently infected with 18537 virus. These targets were barely recognized by the Tc cell line from F VAC-primed mice and less well but significantly lysed by the A2-primed Tc cell line. Our findings indicate that RSV cross-reactivity varied with Tc cells of different viral specificities.

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**Fig. 1.** Induction of Tc cells by priming with VV containing genes for different RSV proteins. Effector Tc cells are spleen cells from mice primed by infection with RSV strain A2 i.n. (●) or vaccinia vectors VF 317 i.n. (■), VF 317 i.p. (○), VN 333 i.p. (□), VAG 301 i.p. (▲), VNG i.p. (●), VLA4 i.p. (○) and VM i.p. (△). RSV-specific target cell lysis represents % lysis of RSV A2-infected P815 cells minus % lysis of uninfected P815 cells. K/T, Killer to target cell ratio.
Fig. 2. Induction of T\textsubscript{c} memory cells by F-VAC and their specificity for the priming virus. T\textsubscript{c} cells were spleen cells from mice primed by infection with (a) RSV A2 i.n. or (b) VF 317 i.p. and restimulated once \textit{in vitro} with the A2 virus. Target P815 cells were uninfected (○), or infected overnight with RSV A2 (●) or 8/60 virus (▲).

Fig. 3. Cross-reactivity pattern, for different RSV strains, of A2- or VF 325-primed T\textsubscript{c} cell lines. T\textsubscript{c} cell lines were restimulated \textit{in vitro} eight times with A2 virus. Target cells were BCH4 cells (persistent Long virus infection) (○), RSV 8/60-infected fibroblasts (▲), RSV A2-infected fibroblasts (△), fibroblasts persistently infected with RSV 18537 (■), or uninfected fibroblasts (□).

**DISCUSSION**

Our results show that RSV F is a significant target antigen for T\textsubscript{c} cells, while T\textsubscript{c} cells are poorly primed by N VAC and not significantly primed by NG VAC, although we used a VV recombinant leading to high expression of N for immunization. In a previous report, we also found only low levels of lysis, in RSV A2-primed BALB/c mice, of target cells infected with N VAC (Bangham \textit{et al.}, 1986), while human T\textsubscript{c} cell lines (several times restimulated \textit{in vitro}) showed strong lysis of N VAC-infected Epstein–Barr virus-transformed syngeneic target cells. We have evidence that there is no problem in expression of N following N VAC infection since N VAC and F VAC induce similar levels of T helper (T\textsubscript{h}) memory cells (P. J. M. Openshaw, R. M. Pemberton, L. A. Ball, G. W. Wertz & B. A. Askonas, unpublished results). Thus, it appears that at least for BALB/c mice, RSV N is not a major RSV target antigen for T\textsubscript{c} cells, but N-specific T\textsubscript{c} cells can be selected by repeated restimulation \textit{in vitro} (M. J. Cannon, unpublished results) and their RSV cross-reactivity will be tested. Thus RSV N may be a better T\textsubscript{c} cell target antigen in man compared to mouse although the results were derived from T cell lines restimulated several times \textit{in vitro} (Bangham \textit{et al.}, 1986). By analogy with the murine T\textsubscript{c} cell response to influenza virus nucleoprotein where there is a high frequency of low responder major histocompatibility complex alleles (Pala & Askonas, 1986), it is possible that other mouse strains will respond better to N VAC.

We find barely significant lysis of RSV-infected target cells following immunization with G VAC, 1A VAC or M VAC which contained only 50\% of the M gene. We know G VAC is expressed since it induced antibodies to G in our mice (P. J. M. Openshaw, unpublished results). Thus, G appears to be a very minor component of the T\textsubscript{c} cell repertoire, but we find that repeated restimulation with RSV \textit{in vitro} can lead to some G-specific T\textsubscript{c} cell selection.
Cytotoxic T cell specificity for RSV

Pala & Askonas (1985) showed that the ability of C57BL mice to induce anti-influenza K\textsuperscript{b}-restricted T\textsubscript{c} cells depended upon the route of inoculation (i.p. or i.n.), as well as the cell type used for restimulation. Whilst recognizing the possibility that the efficiency of T\textsubscript{c} cell-priming in the present experiments may have been influenced by the route of inoculation (though priming i.n. of F VAC was less effective than priming i.p.), or by the presentation of proteins on cells infected with VV, the very high lysis of target cells infected with different RSV strains by mice primed by A2 infection suggests the possibility that other RSV proteins can be recognized by T\textsubscript{c} cells.

Our finding of poor T\textsubscript{c} cell-priming for RSV N in mice, even when we used another N VAC leading to higher N expression than that used in the previous paper (Bangham et al., 1986), contrasted with recognition patterns observed for both influenza virus (Yewdell et al., 1985; Townsend et al., 1984) and VSV (Yewdell et al., 1986), where internal viral nucleoproteins are important target antigens. Thus, T\textsubscript{c} cell recognition patterns vary with different viruses.

Our results also emphasized that the actual protein specificity of the T\textsubscript{c} cells determined the extent of cross-reactivity between antigenically different RSV strains. Certainly, F-specific T\textsubscript{c} cells (following priming of responder mice with F VAC) showed poor cross-reactivity with RSV 8/60 or 18537. In this respect there was concordance with the T\textsubscript{c} cell recognition patterns of influenza virus haemagglutinin (Bennink et al., 1986) and VSV G (Yewdell et al., 1986) as, in all three instances of glycoprotein recognition, T\textsubscript{c} cells appear largely virus strain-specific. However, following infection i.n. with RSV A2, strong cross-reactivity by T\textsubscript{c} cells for RSV 8/60 was seen and an F-specific T\textsubscript{c} cell clone derived from mice primed by RSV A2 infection also recognized 8/60 virus (M. J. Cannon, unpublished results). Thus, F-specific T\textsubscript{c} cells can be RSV cross-reactive or RSV group-specific and immunizing with F VAC may induce a different T\textsubscript{c} cell repertoire compared to infection.

Th cells could also recognize F and this recognition was in part specific for the virus used for priming and in part was cross-reactive for different RSV subgroups (P. J. M. Openshaw, R. M. Pemberton, L. A. Bail, G. W. Wertz and B. A. Askonas, unpublished results). This contrasted with the highly glycosylated G of RSV, which is poorly recognized by T\textsubscript{h} cells and T\textsubscript{c} cells (Bangham et al., 1986). F is also a glycoprotein and expressed at the cell surface, but contains much less oligosaccharide than G (Collins et al., 1984; Wertz et al., 1985).

Immunization of mice with F VAC will induce neutralizing antibody, prevent fusion of RSV-infected cells and protect against RSV infection (Wertz et al., 1987; Olmsted et al., 1986), and monoclonal antibodies specific for F protein will protect mice (Taylor et al., 1984a) and cotton rats (Walsh et al., 1983) from RSV challenge infection. This ability of F to stimulate both humoral and cellular arms of the immune response may make it a possible vaccine candidate.

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