A Bispecific Antibody Recognizing Influenza A Virus M2 Protein Redirects Effector Cells To Inhibit Virus Replication In Vitro

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Bispecific antibodies can be used to redirect cytotoxic T cells to kill virus-infected cells, overriding the need for major histocompatibility complex restriction. We produced a bispecific antibody (3F12) which binds influenza virus M2 protein and the T-cell receptor and can redirect staphylococcal enterotoxin B-activated T cells to kill influenza virus-infected cells and inhibit virus replication in vitro.

Recovery from many viral infections depends on immunity mediated by T cells, including cytotoxic T cells (CD8⁺), which can kill virus-infected cells, and CD4⁺ T cells, which release cytokines such as tumor necrosis factor and gamma interferon. These interactions are major histocompatibility complex (MHC) restricted (reviewed in reference 4) as a result of the T cells' requirement for antigen presentation by class I and class II MHC molecules. In general, antibodies alone have only a secondary role in recovery from virus infections.

We have been exploring the use of bispecific antibodies (BsAbs) to bypass the need for virus-specific MHC-restricted T cells to inhibit experimental virus infections. BsAbs which bind to the T-cell receptor (TCR) as well as a viral protein expressed on the surface of an infected cell can redirect lysis of the infected cell (5, 6). Thus, they can be used as antiviral agents since they combine the specificity of the antibody response with the efficiency of the cellular immune response. An earlier BsAb, HHA6, was constructed to bind the TCR and the hemagglutinin (HA) of influenza A/PR8/34 (PR8) virus (17). HHA6 was shown to be capable of redirecting killing of PR8 virus-infected Bneo cells by staphylococcal enterotoxin B (SEB)-activated T cells, and it could also redirect SEB-activated T cells to inhibit replication of PR8 (H1N1) virus but not the reassortant of A/HK/8/68 and PR8 viruses, X-31 (H3N2) virus, in Madin-Darby canine kidney (MDCK) cells (16). Thus, BsAbs could overcome the need for virus specificity and MHC restriction and inhibit multicycle virus replication. While the BsAbs that we had produced previously were successful, they were made to the HA protein of PR8 virus and therefore were specific for only a single virus strain. One advantage of the use of this technology is that it is possible to generate BsAbs which have wider specificity than neutralizing antibodies or T cells. The influenza virus M2 protein is reported to be very conserved among influenza A viruses (13). It is also reported to be expressed on the surface of infected cells and to function as an ion channel (13, 21). Therefore, we set out to produce a BsAb which would bind M2 with one arm and the TCR with the other and determine if it would function against a wide panel of influenza A virus isolates.

In this paper, we describe the generation of a BsAb (3F12) with these properties. This antibody is cross-reactive for dif-

ferent strains of influenza A viruses and can redirect SEB-activated splenocytes (SEB T cells) to kill influenza virus-infected cells and to inhibit replication of influenza virus in tissue culture.

Generation and screening of BsAbs. BALB/c mice (Charles River Laboratories) were immunized with the keyhole limpet hemocyanin-linked M2 peptide SLLTEVETPIRNEWGCRC ND (13) and boosted with the same peptide 3 weeks later. Splenocytes from these mice were fused by using polyethylene glycol with hypoxanthine-aminopterin-thymidine-sensitive F23.1 cells (15, 24). F23.1 hybridoma produces an immunoglobulin G2a (IgG2a) monoclonal antibody (MAb) with specificity for the V β 8 family of TCR proteins, which is used by 20 to 25% of T cells in BALB/c mice (24).

Supernatants from the hybridomas were tested for the ability to bind the M2 peptide by radioimmunoassay, and a number of positive hybridomas were selected for further experiments. One such hybridoma, 3F12, produced an antibody which could be demonstrated to mediate killing of virus-infected target cells in the presence of V β 8⁺ T cells and was selected for more extensive evaluation. Since the M2 protein of influenza A virus is highly conserved among different strains isolated since 1934, we wanted to test the ability of 3F12 to recognize the extracellular domain of the M2 of as many strains of influenza virus as possible. The binding of 3F12 to different strains of influenza virus was checked by radioimmunoassay using purified viruses to coat the plates and by immunostaining of MDCK cells infected overnight with different strains of influenza virus at a multiplicity of infection (MOI) of 10. By this technique, we detected binding of 3F12 to cells infected with PR8, A/WSN/33 (WSN), A/Udorn/72 (Udorn), A/Japan/305/57 (Japan), and A/Beijing/93 (Beijing) viruses. Figure 1 shows immunostaining of MDCK cells infected with PR8, WSN, Udorn, Japan, and Beijing strains of influenza A virus. MD11 is an anti-M2 MAb used as a positive control (21a). Thus, 3F12 is cross-reactive for different strains of influenza A viruses. The intensity of the staining is weaker for 3F12 than for MD11, which probably results from the fact that 3F12 only has one arm that binds to the M2 protein or from other avidity-related differences between 3F12 and MD11.

Supernatants derived from the 3F12 hybridoma expressed both the IgG2a isotype of F23.1 and an IgG1, which must bind to M2.

Since all BsAbs represent mixtures of parental antibodies, mismatched pairs, and BsAbs (3), we attempted to purify the BsAbs away from the parental antibodies. IgG1 binds poorly to

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FIG. 1. Binding of BsAb 3F12 to cells infected by different strains of influenza virus. Shown is immunostaining of MDCK cells infected overnight with PR8 (A to C), Beijing (D to F), WSN (H to J), Udorn (K to M), and Japan (N to P) influenza viruses at an MOI of 10 or uninfected (G). (A, D, G, H, K, and N) Staining with MD11 (anti-M2 MAb) at 5 µg/ml; (B, E, I, L, and O) staining with BsAb 3F12 at 5 µg/ml. In panels C, F, J, M, and P, medium was added. Triplicate wells were done for each sample with similar results. This figure was generated by the scanning of individual pictures with the program Adobe Photoshop.

protein A, and IgG2a binds strongly; thus, we used a protein A column for purification and eluted with a 0.1 M citrate buffer pH step gradient (between pH 7 and 3.5). All IgG1 parental antibodies passed through the column at pH 6, and IgG2a parental antibodies eluted at pH 4.5. To test which population of the purified antibodies was really bispecific, we performed cytotoxicity assays with influenza virus-infected Bneo cells as targets and SEB T cells as effectors. SEB was used to activate the T cells since it has been shown that it specifically activates T cells expressing Vβ8 TCR proteins (7, 9, 27), which are the only ones bound by our BsAb (16). As shown in Fig. 2, the fraction which eluted at pH 4.9 contained most of the antibody capable of redirecting lysis of PR8-infected cells in a 4-h chromium release assay. This fraction, considered to be the most efficient bispecific fraction, was used for further experiments.

3F12 can redirect SEB T cells to kill influenza virus-infected cells. Bneo cells were infected for 6 h with different strains (PR8, WSN, and Udorn) of influenza virus at an MOI of 5, and then a cytotoxicity assay was performed with SEB T cells as effector cells at an effector-to-target ratio of 20:1. As shown in Fig. 2, 3F12 could redirect SEB T cells to kill cells infected by any of these viruses. On the other hand, HHA6, which is a BsAb specific for the HA protein of PR8 virus and the Vβ8 TCR, could redirect the SEB T cells to kill only PR8 virus-infected cells.

Inhibition of influenza virus multicycle replication in MDCK cells by 3F12. While 3F12 clearly demonstrated the broad specificity predicted for a MAb to the M2 protein, the real test of this antibody was whether it could inhibit multicycle replication of influenza virus in vitro. To test this, we used PR8 virus to infect MDCK monolayers. PR8 virus was chosen be-

FIG. 2. Killing of influenza virus-infected cells by SEB-activated T cells redirected by different fractions of pH-purified 3F12. Supernatants from 3F12 hybridomas were purified through a protein A column, using a pH gradient of citrate buffer (pH 7 to 3.5) to elute. Peak fractions were tested for the ability to redirect SEB-activated T cells to kill influenza virus-infected Bneo cells in a 4-h chromium release assay. PR8 virus was used at an MOI of 5 for 1 h, all antibody fractions were used at 2 μ g/ml (except 3F12, which is supernatant from the hybridomas), and the effector-to-target ratio was 20:1. Data are the averages of triplicate determinations. MED, medium.

cause it replicates more efficiently than other isolates in MDCK cells. MDCK cells were infected with PR8 virus at an MOI of 0.1 and incubated with either 3F12 (8 μ g/ml) or SEB T cells (effector-to-target ratio of 20:1) or both. Supernatants were harvested every 12 h and tested for infectivity by inoculation of MDCK cells, which were left for 48 h. As shown in Fig. 3, 3F12 was able to redirect SEB T cells to inhibit virus replication. Infectivity titers were determined by hemagglutination of 0.5% chicken erythrocytes, using supernatants from the MDCK infectivity. Endpoint virus titers were determined by interpolation of the dilution that infected 50% of the wells by the method of Reed and Muench (22). As seen in Fig. 4, virus titers in supernatants were reduced by 2 $\log_{10}$ units when 3F12 and SEB T cells were present compared with the titers obtained in the presence of T cells alone. This inhibition of virus replication was maintained throughout the infection. It should also be noted that 3F12 was unable to inhibit virus replication by itself.

It has been convincingly demonstrated that BsAbs which bind to the TCR of a cytotoxic T lymphocyte with one arm and a tumor target cell with the other can trigger the cytotoxic T lymphocyte to kill the tumor, a process known as redirected lysis (8, 12, 19, 20, 25, 26). We and others have shown that BsAbs generated by hybridoma fusion (11, 14, 15, 28) can redirect T cells to kill virus-infected cells, even though the T cells are not virus specific (1, 10, 18, 17, 24). In addition, we have documented that cytotoxic T lymphocytes and bifunctional antibodies can inhibit multicycle virus replication when added to cultures of infected cells (16). These findings have suggested the possibility that BsAbs can be used in an immunotherapeutic protocol against virus infections in vivo.

3F12 can redirect superantigen-activated T cells to kill virus-infected target cells, again demonstrating broad cross-reactivity with various virus subtypes. The crucial test was its ability to inhibit multicycle virus replication in virus-infected target cells. We demonstrate in this report that 3F12 efficiently does so. We are presently attempting to use this BsAb to inhibit virus replication and speed recovery of animals infected with influenza virus.

In these experiments, we used as effector cells superantigen-activated T cells and SEB in particular, since all of our BsAbs have one arm specific for the V β 8 TCR. Thus, in a short 3-day incubation, we can produce large numbers of activated V β 8⁺ T cells, which are a combination of CD8⁺ killer cells and CD4⁺ cells producing cytokines (primarily gamma interferon). The mechanism by which these cells inhibit multicycle virus replication is unclear. We demonstrated in a previous publication that both CD4⁺ and CD8⁺-enriched T cells are capable of inhibiting virus replication (16). This finding parallels the results obtained by Doherty et al. demonstrating that both subpopulations have the potential to clear virus in an infected animal (4).

Although we do not anticipate that immunotherapeutic approaches will prove useful against influenza, we are anxious to explore such a strategy as a convenient experimental model of immunotherapy in vivo. It is our contention that the use of superantigen-activated T cells and BsAbs is simpler and more convenient than the generation of large numbers of antigen-specific T-cell clones currently used in some forms of antiviral immunotherapy (23). We envision that ultimately, a patient's own T cells will be collected and activated ex vivo, incubated with the appropriate anti-TCR, antiviral protein or tumor

FIG. 3. 3F12 redirects SEB T cells to kill cells infected by different strains of influenza virus. Bneo cells were infected (MOI of 5) for 6 h with PR8, WSN, or Udorn virus. A 4-h ^{51}Cr release assay was performed in which HHA6 and 3F12 were used at 2 $\mu\text{g}/\text{ml}$ and SEB T cells were used at an effector-to-target ratio of 20:1. Means of triplicates are shown, and the standard deviations are 1.32 (mock plus HHA6), 1.27 (mock plus 3F12), 4.59 (mock), 0.39 (PR8 plus HHA6), 1.76 (PR8 plus 3F12), 0.63 (PR8 plus mock), 5.26 (WSN plus HHA6), 7.1 (WSN plus 3F12), 2.38 (WSN plus mock), 1.33 (Udorn plus HHA6), 6.9 (Udorn plus 3F12), and 2.32 (Udorn plus mock).

BsAb, and returned to the patient. If such cells can destroy a fraction of the virus-infected or tumor cells, they may function to help immunosuppressed or immunocompromised patients cope more efficiently with an ongoing or persistent viral infection or tumor. In addition to the ability of these cells to kill virus-infected cells, they (particularly the CD4^+ population) may also function to release cytokines at the site of virus

infection, which may aid in recovery. Chisari and Ferrari (2) have clearly demonstrated that in their mouse hepatitis B virus model, inhibition of virus transcription is dependent on cytokine release from activated T cells rather than cytotoxicity. Thus, chronically infected hepatitis B virus-infected patients may be induced to recovery by targeting of cytokine-secreting T cells to infected livers. Even in patients infected with human immunodeficiency virus, it may be possible to infuse an antibody bearing superantigen-stimulated autologous CD8^+ T cells into patients to decrease the numbers of infected cells. If we can show experimentally that BsAbs can be used in vivo, we would like to create BsAbs, such as the one described in this report, which have broad cross-reactivity for as many isolates as possible.

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FIG. 4. 3F12 can redirect SEB T cells to inhibit influenza virus replication in tissue culture. MDCK cells were infected with PR8 virus (MOI of 0.1) and incubated for 48 h in the presence of medium (Δ), 3F12 ($\square$), SEB T cells ($\blacktriangle$), or both ($\blacksquare$). Samples were taken every 12 h and used for infectivity in MDCK cells for another 48 h. Supernatants were tested for virus titers by hemagglutination of chicken erythrocytes. Triplicate determinations were done for each sample, and mean values are shown in the curve. The difference in inhibition of virus replication by 3F12 plus SEB T cells compared with SEB T cells alone was statistically significant after 12 h by a two-tailed *t* test, with *P* values of 0.006 (24 h), 0.01 (36 h), and 0.002 (48 h).

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