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ISOLATION AND RECOMBINANT EXPRESSION OF AN MHV-JHM NEUTRALISING MONOCLONAL ANTIBODY

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1. ABSTRACT

The monoclonal antibody A1 (mab A1) efficiently neutralises the infection of susceptible cells by the murine hepatitis virus MHV-JHM in vitro and in vivo (Wege et al., 1984). The variable regions of mab A1 were amplified from mRNA of the respective hybridoma cell line by RT-PCR and integrated into different eukaryotic expression vectors. The biological function of the recombinant antibody constructs was verified by virus neutralisation assays. Whereas a complete recombinant antibody (mab A1rec.) expressed in transfected murine myeloma cells inhibited the MHV-JHM infection as well as the parental antibody, a single-chain Fv derived from mab A1 did not show any neutralising activity.

2. INTRODUCTION

Coronaviruses are enveloped, positive-stranded RNA viruses causing diseases of economical importance in animals and humans. Generally, coronavirus induced diseases show a pronounced pathogenicity in new-born animals. The marked susceptibility of new-born animals is presumably a consequence of their still immature immune system. Lactotropic transfer of maternal, antiviral antibodies via the placenta and milk efficiently protects the new-born animals against fatal consequences of acute coronaviral infections. Administration of neutralising antibodies via an intraperitoneal injection and also by oral application has emerged as potential tool of interference with coronavirus infections in offspring of non-immune mothers (Wege et al., 1984; Enjuanes and van der Zeijst, 1995).

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Generation of transgenic animals expressing neutralising antibodies directed against coronaviral pathogens in the lactating mammary gland may therefore provide new-born animals with an efficient protection against fatal infections during a critical period.

The murine hepatitis virus (MHV) strain JHM leads to fatal neurological disorders, especially in new-born animals. MHV-JHM infections can be efficiently inhibited in vitro and in vivo by neutralising antibodies and CD4+ T-cells (Wege et al., 1984; Flory et al., 1993). The majority of neutralising mabs are directed against the viral surface glycoprotein (S). Mab A1 (Wege et al., 1984) is one of the most potent antibodies with regard to virus neutralisation and inhibition of virus induced cell-to-cell fusion. It interacts with the S1 subunit of the MHV-JHM S-protein. We have isolated the variable regions of mab A1 and transferred them into different eukaryotic expression vectors. The biological function of the recombinantly expressed antibodies was assessed by MHV-JHM neutralisation assays.

3. MATERIALS AND METHODS

3.1. Cells and Viruses

DBT cells were grown as described (Kolb et al., 1996) NS0 mouse myeloma cells (NS0, ECACC 85110503) were cultivated at 37°C in RPMI 1640 medium (Sigma, Deisenhofen, Germany) supplemented with 10% FBS, non-essential amino acids, glutamine and antibiotics. The virus strain used for these studies was described previously (Routledge et al., 1991). Transfections were done as described (Kolb and Siddell, 1996). For virus neutralisation assays 500µl of the respective MHV-JHM dilution was mixed with 500µl of tissue culture supernatant from antibody expressing cells or control cells and incubated for 1h at 37°C. Subsequently, the virus-antibody mixture was added to confluent DBT cells and incubated for one further hour at 37°C. The virus containing supernatant was then removed. The cells were washed twice with PBS and overlaid with cell culture medium containing 1% agarose. Virus plaques were counted after an incubation period of 16h at 37°C.

3.2. DNA Cloning

The isolation and cloning of the RT-PCR products corresponding to the variable regions of the mab A1 heavy and light chain cDNAs was performed as described (Orlandi et al., 1989). The single-chain Fv derived from mab A1 consists of 1. a 216bp fragment of the murine Metallothionein promoter, 2. 400bp of the human growth hormone (hGH) gene encoding the hGH signal peptide, 3. the mab A1 heavy and light chain variable regions, 4. an 38nm spacer region (3 consecutive stretches of the amino acid sequence GGGGS; Huston et al., 1988) which is inserted between the heavy and light chain variable domains, 5. a peptide tag recognised by the monoclonal antibody 30B (Routledge et al., 1991) (amino acid sequence: IPRSRQIDLQIG) (Fig. 3a) and 6. 275bp of the 5th exon of the hGH gene containing the polyadenylation signal. The protein encoding DNA fragments were assembled in one open reading frame which encodes a protein with a predicted molecular mass of 26500 Da.

3.3. Protein Analysis

Western blot analyses were performed as described (Kolb et al., 1996). Development of the blots was done by using 4-Chloronaphtol or the Amersham ECL system.

4. RESULTS

4.1. Isolation of the Variable Regions of Mab A1

The monoclonal antibody A1 efficiently neutralises the infection of MHV-JHM into susceptible cells. To express a recombinant antibody with the same biological properties we isolated the variable regions of the mab A1 by reverse transcription and PCR by using degenerate primers corresponding to conserved regions of the variable domains (Orlandi et al., 1989). The sequence of the mab A1 heavy and light chain variable regions are available under the gene bank accession numbers AF 001575 and AF 001576, respectively. The PCR products of 322bp and 347bp derived from the light and heavy chain cDNAs were subsequently cloned into the expression vectors Lys17 and Lys30 (Orlandi et al., 1989), respectively. These vectors carry IgG constant domains of human origin. Additionally, the vectors contain the hygromycin-phosphotransferase and the xanthin-guanosine-phosphoribosyl-transferase genes as selectable markers.

4.2. Recombinant Expression of the Mab A1

The expression plasmid Lys17-A1L was then transfected into NS0 myeloma cells. After selection in medium containing 100µg/ml hygromycin B, 10 of the 52 resulting cell clones were analysed for the expression of the IgG kappa chain. 8 of the 10 cell clones secreted high amounts of light chain protein into the tissue culture supernatant. One of these clones was then re-transfected with the plasmid Lys30-A1H. After selection in medium containing 1µg/ml mycophenolic acid one cell clone (designated NS0 Lys17-A1L/Lys30-A1H) could be recovered which expressed detectable amounts of heavy chain (Fig. 1). The amount of light chain protein present in the supernatant clearly exceeds the amount of heavy chain (Fig. 1). However, after purification of the supernatant over a protein G-sepharose column equimolar amounts of heavy and light chain protein can be detected in a Western blot analysis (Fig. 1). Comparison to a human IgG protein standard reveals that NS0 Lys17-A1L/Lys30-A1H cells (1×10^7 cells in 14 days) can accumulate to about 3mg/ml of recombinantly expressed light chain in their supernatant (Fig. 1). The amount of heavy chain protein appears to be tenfold lower (Fig. 1).

The biological activity of the recombinantly expressed antibody (mab A1rec.) was analysed by virus neutralisation assays. The tissue culture supernatant of NS0 cells transfected with the parental plasmids Lys17 and Lys30 encoding an irrelevant antibody was used as a negative control. As expected, only the supernatant of NS0 Lys17-A1L/Lys30-A1H cells was capable of neutralising MHV-JHM infection of DBT cells (Fig. 2a). The efficiency of virus neutralisation was compared between the supernatants of A1 hybridoma cells and NS0 Lys17-A1L/Lys30-A1H cells (Fig. 2b). The plaque reduction efficiency was in the same order of magnitude for both supernatants indicating that the correct variable region were cloned and expressed.

4.3. Expression of an scFv Derived from Mab A1

We next investigated whether a single-chain Fv (scFv) fragment carrying the mab A1 variable regions would also possess the highly neutralising activity of its parental counterpart. Single-chain Fvs offer several advantages over complete antibody molecules, in that they are made up of only one peptide chain, so that only one transcriptional unit would have to be transferred in a transgenic approach. Inherent with the nature of the scFv

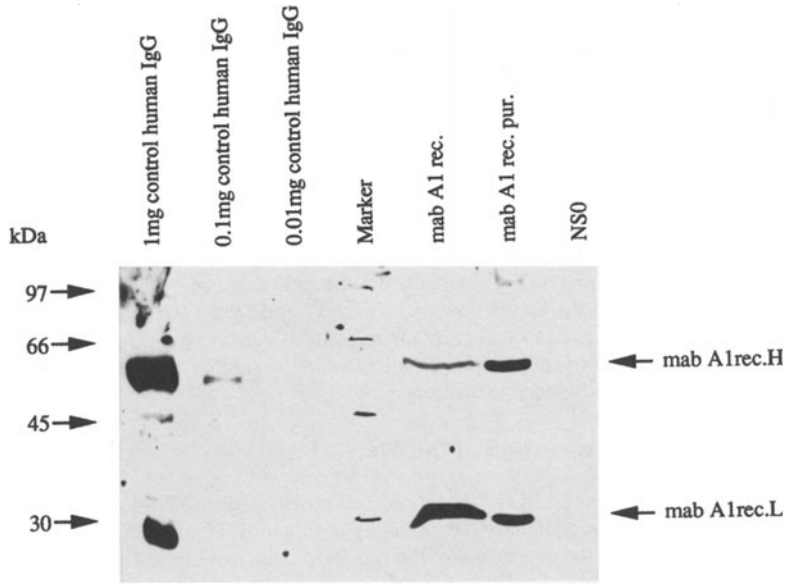

Figure 1. Western blot analysis of the recombinantly expressed mab A1 (mab A1rec.). Supernatants of NS0 cells and NS0 cells stably transfected with the expression vectors Lys17-A1L and Lys30-A1H and 3 different dilutions of a human IgG protein standard (Behring-Werke, Marburg, Germany) were separated on a 15% polyacrylamide gel and blotted to nitro-cellulose. The molecular masses of the molecular weight marker proteins and the positions of the IgG heavy (mab A1rec.H) and light chain proteins (mab A1rec.L) are indicated.

molecule, heavy and light chain sequences are present in equimolar amounts. Finally, scFvs may also be produced in bacterial expression systems, so that large amounts of an antiviral compound can be synthesised.

We generated an scFv expression cassette (Fig. 3a) in which the heavy and light chain variable regions of mab A1 were separated by a linker consisting of 3 consecutive stretches of the amino acid sequence GGGGS (Huston et al., 1988). In order to allow for the secretion of the scFv, the human growth hormone (hGH) signal peptide (sp) was linked to the N-terminus of the predicted peptide chain. A peptide tag recognised by the monoclonal antibody 30B (Routledge et al., 1991) was added at the C-terminus of the scFv open reading frame to permit the immunological detection of the protein. The expression cassette was linked the strong constitutive murine Metallothionein (MT) promoter and the hGH polyadenylation signal (Fig. 3a). The expression vector (designated MThGH-A1scFv-tag) was transfected into DBT cells together with the plasmid pSV2-neo carrying a selectable neomycin-phosphotransferase marker gene. After selection in medium containing 400μg/ml G418, the resulting clones were analysed for the expression of the A1-scFv by Western blotting. The highest amounts of the A1-scFv could be detected in cytoplasmic extracts and the supernatant of the cell clone DBT MThGh-A1scFv-tag-6 (Fig. 3b).

Again the biological activity of the recombinantly expressed antibody derivative was assessed by a virus neutralisation assay. In a first experiment DBT MThGh-A1scFv-

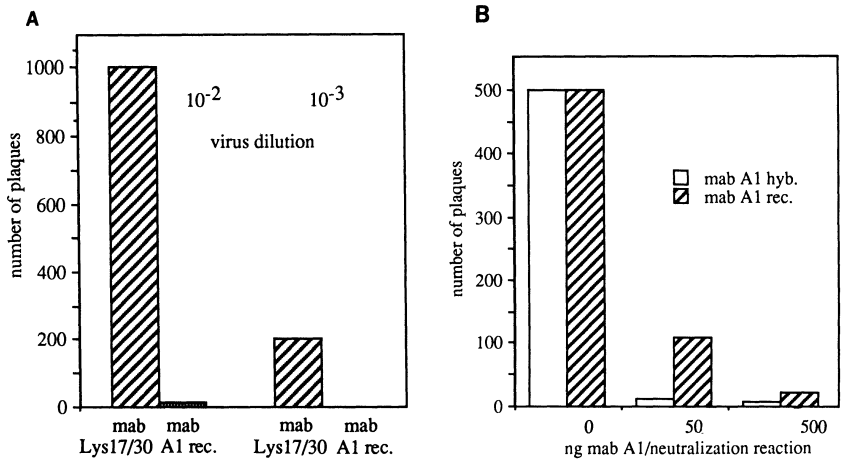

Figure 2. Virus neutralisation assay. A) Tissue culture supernatants derived from the cell clone NS0 Lys17-A1L/Lys30-A1H or the negative control cell clone NS0 Lys17/Lys30 (stably transfected with the plasmids Lys17 and Lys30 which encode an irrelevant mab) were analysed in a virus neutralisation assay. The number of virus plaques obtained in a representative experiment with virus dilutions of 10^{-2} and 10^{-3} are presented. B) The supernatants of A1 hybridoma and NS0 Lys17-A1L/Lys30-A1H cells were diluted in MEM cell culture medium. The amounts of antibody present in the supernatant was estimated by calibration against murine and human antisera in a Western blot assay. The number of virus plaques obtained are presented in relation to the total amount of mab present in the supernatants applied to the neutralisation reaction.

tag-6 cells were infected with different dilutions of MHV-JHM. As a negative control untransfected DBT cells and DBT cells transfected with the control plasmid pBK-CMV (Stratagene) were infected in parallel. However, no differences in the number and size of virus-induced plaques could be detected (Fig. 3c). This suggests that the intracellular expression of the A1-scFv does not lead to an inhibition of MHV-JHM infection. One reason for this result might be that the A1-scFv is not present in the same cellular compartments as the MHV-JHM surface glycoprotein (S) and therefore fails to inhibit the S-protein function in virus entry and virus maturation. We therefore analysed the ability of the secreted A1-scFv to neutralise MHV-JHM infection. The supernatant of DBT MThGh-A1scFv-tag-6 cells, however, was also unable to inhibit MHV-JHM entry into DBT cells (Fig. 3d).

5. DISCUSSION

We have isolated the variable regions of a monoclonal antibody which neutralises MHV-JHM infections into susceptible cells. A recombinant version of the mab A1 was successfully expressed in NS0 myeloma cells. An scFv carrying the variable regions of mab A1, however, failed to inhibit MHV-JHM infection, indicating that an scFv derived from a monoclonal antibody does not necessarily display the same biological function. A number of reasons could account for the inability of the A1-scFv to neutralise MHV-JHM and future experiments will preferentially address the question as to whether the A1-scFv is able to physically interact with the MHV-JHM surface glycoprotein.

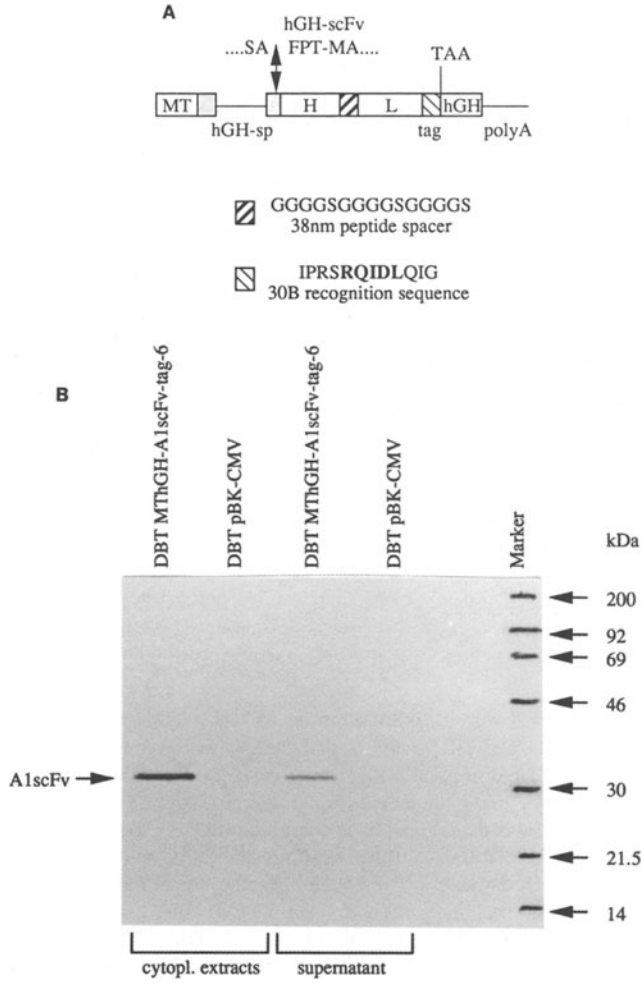

Figure 3A and B. A) Schematic representation of the single-chain Fv derived from mab A1. The murine Metallothionein promoter (MT), heavy (H) and light chain (L) variable regions and the 5th exon of the human growth hormone (hGH) are marked as open boxes. The hGH signal peptide, the 38nm spacer segment and the 30B amino acid tag sequence are represented as dotted, heavily striped and striped boxes, respectively. The translational stop codon (TAA) and the hGH polyadenylation signal (polyA) are indicated. The junction between the hGH signal peptide and the scFv open reading frame is marked with a dash (-). The site at which the hGH signal peptide is predicted to be cleaved is marked with a double arrow. B) Western blot analysis of A1 scFv expression. Cytoplasmic extracts and tissue culture supernatants of DBT cells transfected with pBK-CMV and of the cell clone DBT MThGh-A1scFv-tag-6 carrying the A1 scFv expression plasmid were separated on a 15% polyacrylamide gel and blotted to nitro-cellulose. scFv protein was detected by using the antibody 30B.

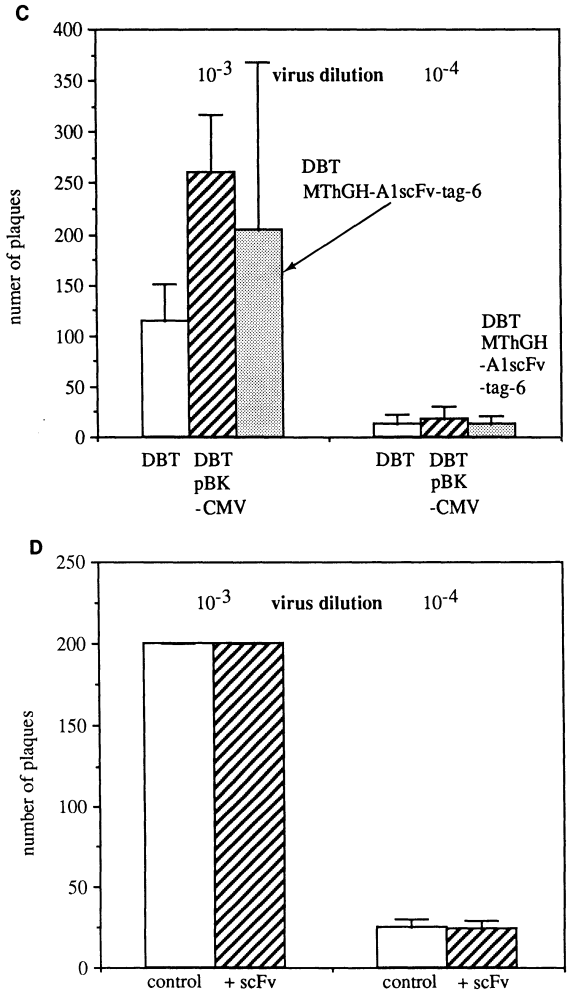

Figure 3C and D. C) Virus neutralisation assay. DBT cells, DBT cells transfected with the plasmid pBK-CMV and DBT MThGh-A1scFv-tag-6 cells were analysed for their susceptibility to MHV-JHM infection. The average number of virus plaques scored after 3 independent infections with 2 different virus dilutions are presented. D) Virus neutralisation assay. The supernatants of DBT pBK-CMV cells and DBT MThGh-A1scFv-tag-6 cells were analysed for ability to neutralise MHV-JHM infection into DBT cells. The average number of virus plaques scored after 3 independent infections with 2 different virus dilutions are presented.

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