Neutralizing human monoclonal antibodies against Puumala virus, causative agent of nephropathia epidemica: a novel method using antigen-coated magnetic beads for specific B cell isolation

Åke Lundkvist, Jan Hörling, Leif Athlin, Anders Rosén and Bo Niklasson

Introduction

Hantaviruses, a separate genus in the Bunyaviridae family, are the causative agents of several human diseases with similar clinical symptoms. The diseases, commonly called haemorrhagic fever with renal syndrome (HFRS), are characterized by fever and renal dysfunction and, in severe cases, haemorrhagic manifestations (Yanagihara & Gajdusek, 1988). The genomes of hantaviruses consist of three negative-stranded RNA segments, which encode four structural proteins: a large protein (L), two envelope glycoproteins (G1 and G2) and a nucleocapsid protein (N) (Elliott, 1990; Sheshberadaran et al., 1988). Several species of small rodents are natural reservoirs of hantaviruses and transmission to humans occurs via aerosolized excretions (Elliott, 1990). Hantaviruses isolated from different hosts can be differentiated into at least five serotypes based on the patterns of neutralization, immunofluorescence and immunoprecipitation assays (Schmaljohn et al., 1985; Lee et al., 1985; Sheshberadaran et al., 1988; Baek et al., 1988). The representatives of each serotype are Hantaan, Seoul, Puumala (PUU), Prospect Hill and Leakey viruses, respectively (Yanagihara & Gajdusek, 1988; Baek et al., 1988).

Nephropathia epidemica (NE), a form of HFRS that occurs in Scandinavia, Finland, western Russia and central Europe, is caused by PUU virus or closely related virus strains (Lähdevirta, 1971; Brummer-Korvenkontio et al., 1980; Sheshberadaran et al., 1988). NE occurs sporadically in central Europe but causes significant morbidity in Scandinavia, Finland and Russia. The annual incidence in many endemic regions exceeds 100 cases per 100000 inhabitants (Niklasson et al., 1993). The majority of all recorded cases are hospitalized for a few weeks and the overall fatality rate is 0-1 to 1% (Settergren et al., 1991). No vaccine or specific therapy for NE is currently available.

Human monoclonal antibodies (MAbs) against Puumala (PUU) virus were generated and characterized. Human spleen B lymphocytes were preselected for specific surface immunoglobulin (Ig) using magnetic beads coated with the viral glycoproteins, and subsequently immortalized by Epstein–Barr virus transformation. Four IgG-positive monoclonal lymphoblastoid cell lines (LCLs) were established and have remained stable MAb secretors for over 12 months. Analyses of the antigen and epitope specificities recognized by the MAbs showed overlapping binding patterns of four antiglycoprotein 2-specific clones. Identical isotypes (IgG1) and isoelectric points (9.2) of the four MAbs suggested that they were derived from the same original clone. The MAbs reacted with eight PUU virus-like strains, but were negative for Hantaan, Seoul, and Prospect Hill viruses in an immunofluorescence assay, indicating binding to a conserved epitope unique for strains associated with the European form of haemorrhagic fever with renal syndrome, nephropathia epidemica. The MAbs neutralized all investigated PUU virus-like strains in a focus reduction neutralization test. The MAb neutralizing activity was significantly enhanced in the presence of human or guinea-pig complement. To stabilize and increase antibody secretion and to reduce the demand for culture medium supplements (e.g. fetal calf serum), three of the monoclonal LCLs were fused with the non-secreting human × mouse partner SPAM-8. Several of the established human × (human × mouse) monoclonal triomas grew faster and produced larger amounts of MAbs when compared with the original LCLs.
Protein G-Sepharose (pG-S) columns (Pharmacia).

Methods

Cells and viruses. Human spleen lymphocytes were obtained from a 60-year-old female patient splenectomized because of idiopathic thrombocytopenic purpura. The lymphocyte fraction was isolated on Lymphoprep according to the manufacturer's instructions (Nygaard) and cryopreserved at -70 °C or in liquid nitrogen.

Bank vole MAbs 1C12, 5A2, 4G2 and 5B7, specific for PUU virus N, G1, G2a and G2b, respectively (Lundkvist & Niklasson, 1992), were affinity-purified from culture supernatants on Protein G-Sepharose (pG-S) columns (Pharmacia).

The history of the hantavirus strains used in this study is summarized in Table 1. All virus strains were propagated in Vero E6 cells (CRL 1586; ATCC) cultivated in Eagle's MEM (Gibco) supplemented with 10 % fetal calf serum (FCS), 2 mM-L-glutamine, 60 μg/ml penicillin and 100 μg/ml streptomycin.

Table 1. History of virus strains and immunofluorescence reactivity of MAb 1C9

<table>
<thead>
<tr>
<th>Virus strain</th>
<th>Species</th>
<th>Location</th>
<th>Reference</th>
<th>IFA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Puumaala Sotkamo</td>
<td>Clethrionomys glareolus</td>
<td>Finland</td>
<td>Schmaljohn et al. (1985)</td>
<td>256*</td>
</tr>
<tr>
<td>83-L20</td>
<td>Clethrionomys glareolus</td>
<td>Sweden</td>
<td>Isolated from C. glareolus (lung) trapped 1983, Hallnäs</td>
<td>64</td>
</tr>
<tr>
<td>CG-13891</td>
<td>Clethrionomys glareolus</td>
<td>Belgium</td>
<td>Van der Groen et al. (1987)</td>
<td>256</td>
</tr>
<tr>
<td>CG-1820</td>
<td>Clethrionomys glareolus</td>
<td>Russia</td>
<td>Tkachenko et al. (1984)</td>
<td>64</td>
</tr>
<tr>
<td>P-360</td>
<td>Human</td>
<td>Russia</td>
<td>Kindly provided by Dr E. A. Tkachenko</td>
<td>256</td>
</tr>
<tr>
<td>K-27</td>
<td>Human</td>
<td>Russia</td>
<td>Sheshberadaran et al. (1988)</td>
<td>64</td>
</tr>
<tr>
<td>H-45</td>
<td>Human</td>
<td>Russia</td>
<td>Kindly provided by Dr E. A. Tkachenko</td>
<td>64</td>
</tr>
<tr>
<td>Hantaan (76-118)</td>
<td>Apodemus agrarius</td>
<td>Korea</td>
<td>Lee et al. (1978)</td>
<td>-†</td>
</tr>
<tr>
<td>Seoul (80-39)</td>
<td>Rattus norvegicus</td>
<td>Korea</td>
<td>H. W. Lee et al. (1982)</td>
<td>-</td>
</tr>
<tr>
<td>Prospect Hill</td>
<td>Microtus pennsylvanicus</td>
<td>U.S.A.</td>
<td>P. W. Lee et al. (1982)</td>
<td>-</td>
</tr>
</tbody>
</table>

* Reciprocal IFA titres of cell culture supernatants.
† -, Negative.

We have recently described the establishment of bank vole–mouse heterohybridomas secreting neutralizing monoclonal antibodies (MAbs) against PUU virus. It was shown that both viral envelope glycoproteins expressed neutralizing domains (Lundkvist & Niklasson, 1992). However human MAbs are preferable to rodent MAbs for studies of the human immune repertoire and for potential immunotherapy.

In the present study, we generated and characterized human MAbs specific for PUU virus G2 by using a novel method involving magnetic beads for specific B cell isolation. The MAbs were examined for reactivities and neutralizing activities against several PUU virus-like strains isolated in different parts of Europe.

Strategies for generation of human MAbs to PUU virus glycoproteins. Four strategies for the establishment of human MAb-secreting cell lines were evaluated. We compared two previously published methods [(i) and (ii)] with two novel approaches [(iii) and (iv)]. Epstein-Barr virus (EBV) transformation of unselected B lymphocytes followed by cloning by limiting dilution (Rosén et al., 1983); [ii] fusions of unselected B cells with human (KR4; Kozbor et al., 1982) and mouse-human (SPAM-8; Gustafsson et al., 1991) fusion partners followed by cloning. (iii) EBV transformation followed by selection of immortalized specific B cells with virus glycoprotein-coated magnetic beads, and (iv) preselection of specific B cells by magnetic beads followed by EBV transformation and cloning.

Antigen-specific preselection of human B cells by PUU virus glycoprotein-coated magnetic beads. One-hundred μg of affinity-purified anti-G1 and anti-G2 bank vole MAbs, 5A2 and 5B7 respectively, were incubated with 104 magnetic beads coupled with goat anti-mouse IgG according to the manufacturer’s instructions (Dynal). After five washes in PBS containing 1 % FCS, the beads were subsequently incubated overnight with PUU virus antigen (strain 83-223L), prepared as previously described (Lundkvist & Niklasson, 1992), diluted twofold in PBS, followed by five washes in PBS containing 1 % FCS. The principle of the cell separations is illustrated in Fig. 1. Spleen cells were washed twice with cold RPMI-1640 containing 10 % FCS prior to incubation with beads (cell-bead ratio of 10:1). Rosetting was performed for 2 h at 4 °C in the same medium with gentle agitation of the suspension in an end-over-end rotator. The rosetted cells were separated from non-rosetted cells by placing the tube in a magnetic device (MPC-1; Dynal) for 90 s. The magnet-bound cells were washed five times with 10 ml cold medium by resuspension, followed by further magnetic separation. The percentage of magnet-separable rosettes was determined by counting cells under the microscope.

Establishment of monoclonal EBV-transformed lymphoblastoid cell lines (LCLs). Antigen-selected B lymphocytes were immortalized by cocultivation with supernatant from the EBV-infected marmoset cell line B95-8 (Miller & Lipman, 1973) and incubated on irradiated (3000 rad) human fibroblast feeder cells (RHF) in Iscove's medium–Opti-MEM (1:1) (Gibco) with 15 % FCS. One week later, the cells were cloned by limiting dilution (100 cells/96-well plate) on RHF in
Neutralizing human MAbs against PUU virus

washing, serial two-fold dilutions of the samples and a purified IgG1 λ standard (The Binding Site) were incubated for 1 h. Alkaline phosphatase-conjugated goat anti-human IgG (γ-chain specific) (Sigma), followed by p-nitrophenyl phosphate (Sigma), were used to detect specific antibody binding.

Focus reduction neutralization test (FRNT). Neutralization titres of the established MAbs were examined in FRNT as described elsewhere (Niklasson et al., 1991). In short, samples were serially diluted and mixed with equal volumes of approximately 50 f.f.u. of PUU virus for 1 h, before incubation on Vero E6 cells. After adsorption for 1 h, the cells were overlaid with 0.5% agarose and incubated for 14 days. The agarose layer was removed and the cells were fixed with methanol. Viral antigen was visualized by addition of rabbit anti-PUU virus serum followed by peroxidase-conjugated goat anti-rabbit IgG and substrate. The neutralization activity of an antibody was expressed as the amount of IgG necessary to reduce the number of foci by 80%.

Epitope mapping. Epitope specificities of MAbs were determined by a competitive ELISA. Microtitre plates were coated with bank vole anti-G2 MAb 5B7, followed by blocking and virus antigen as described above for PUU virus G2-specific ELISA. After washing, 100 µl/well of each of the pG-S-purified human MAbs and the bank vole MAb 4G2 (diluted to 10 µg/ml in ELISA buffer) were incubated in duplicate for 1 h at 37°C. After washing, 100 µl/well of the same MAbs, biotinylated as previously described (Harlow & Lane, 1988) and diluted to 0.1 µg/ml, were added to the plates and incubated for 1 h at 37°C. Binding of biotinylated antibody was detected by alkaline phosphatase-conjugated Extravidin followed by p-nitrophenyl phosphate, according to the manufacturer’s instructions (Sigma).

Immunofluorescence assay (IFA). Spot slides with virus-infected Vero E6 cells were prepared and stained as described earlier (Niklasson & LeDuc, 1987). Briefly, PPUU-related virus strains were incubated in Vero E6 cultures for 14 days before applying the cells to the microscope slides. Hantaan, Seoul and Prospect Hill viruses were incubated for 9, 10 and 8 days, respectively. MAb supernatants were incubated for 60 min. Fluorescein isothiocyanate-conjugated sheep anti-human IgG (Kirkegaard) diluted 1:10 was used for detection of virus-specific antibodies.

Immunoblotting. The MAbs were examined for their reactivity with virus strain 83-223L in an immunoblot, as previously described (Lundkvist et al., 1991).

Results

Generation of human MAbs

The serum of the spleen donor had reciprocal ELISA IgG endpoint titres (defined as twice the absorbance of a negative serum at the same dilution) of 6400, 1600 and 6400, against PUU virus N, G1 and G2, respectively.
Table 2. Specificities of human MAbs determined by competitive ELISA*

<table>
<thead>
<tr>
<th>Biotin-labelled MAb</th>
<th>Competing MAb</th>
<th>1C9</th>
<th>1E2</th>
<th>2E8</th>
<th>2F10</th>
<th>4G2</th>
</tr>
</thead>
<tbody>
<tr>
<td>1C9</td>
<td>0.00</td>
<td>0.01</td>
<td>0.01</td>
<td>0.03</td>
<td>0.40</td>
<td></td>
</tr>
<tr>
<td>1E2</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.03</td>
<td>0.41</td>
<td></td>
</tr>
<tr>
<td>2E8</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.02</td>
<td>0.39</td>
<td></td>
</tr>
<tr>
<td>2F10</td>
<td>0.00</td>
<td>0.01</td>
<td>0.00</td>
<td>0.03</td>
<td>0.42</td>
<td></td>
</tr>
<tr>
<td>4G2</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.01</td>
<td></td>
</tr>
</tbody>
</table>

* Inhibition of binding of biotin-labelled MAb by unlabelled MAbs. Results are expressed as absorbances relative to that without competition (diluent only).

Reciprocal IFA titres were 160 and 40 against strains 83-223L and 76-118, respectively. No virus-specific IgM could be detected. The neutralizing antibody titre against strain 83-223L was 640, as determined by FRNT.

Four different strategies for production of PUU virus-specific human MAbs were employed. (i) Unselected spleen B cells were EBV-transformed and seeded on RHF in 96-well plates, 100 cells/plate. Growing clones (134) were screened for PUU virus-specific antibodies by ELISA. No positive wells were found. (ii) B cells (1.6 x 10⁶) were fused with the fusion partners KR4 or SPAM-8. The fused cells were grown in cell culture flasks (batch culture) in HAT medium for 14 days followed by cloning on RHF. Fifty-six KR4 hybrid clones were screened in ELISA without detection of any antibody-positive wells. Seven weakly IgG-positive wells were found among the 485 SPAM-8 hybrids. Positive cells were subcloned several times but no stable lines could be established. (iii) As a third strategy, unselected cells were EBV-transformed, grown for 21 days in batch culture, and selected by PUU virus glycoprotein-coated magnetic beads. One percent of the cells which remained attached to the beads after washing were transferred to RHF in 24-well plates. Seven days later the supernatants were tested for virus-specific IgM and IgG. Several IgG-positive wells were found and cloned on RHF in 96-well plates. None of the growing clones were positive when examined 3 to 4 weeks later. (iv) Positive results were obtained when spleen cells were preselected by viral glycoprotein-coated magnetic beads, EBV-transformed and grown on RHF in 24-well plates for 10 days followed by cloning in 96-well plates. Three weeks after cloning, 21 wells contained growing clones. Twenty clones were IgG-positive against PUU virus G2 and three of these also showed a weak reaction against G1. None of the wells contained N-reactive antibodies. None of the wells had detectable levels of virus-specific IgM. Cells from four wells were successfully subcloned and produced G2-specific IgG. These four wells were also found to contain neutralizing antibodies as determined in NT-ELISA. No G1-specific clones could be established. Four clones, designated 1C9, 1E2, 2E8 and 2F10, secreting MAbs specific for G2, were subcloned two to three times to ensure that they were monoclonal, and were further characterized.
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Table 3. Neutralizing activity of human MAb 1C9

<table>
<thead>
<tr>
<th>Virus strain</th>
<th>NT-ELISA without C</th>
<th>NT-ELISA with 1% C</th>
<th>FRNT with 10% C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Puumala Sotkamo</td>
<td>1.05</td>
<td>0.066</td>
<td>0.024</td>
</tr>
<tr>
<td>83-223L</td>
<td>1.05</td>
<td>0.260</td>
<td>0.024</td>
</tr>
<tr>
<td>CG-13891</td>
<td>0.026</td>
<td>0.066</td>
<td>NT†</td>
</tr>
<tr>
<td>CG-1820</td>
<td>0.260</td>
<td>0.066</td>
<td>0.024</td>
</tr>
<tr>
<td>P-360</td>
<td>0.260</td>
<td>0.066</td>
<td>0.024</td>
</tr>
<tr>
<td>K-27</td>
<td>0.260</td>
<td>0.066</td>
<td>0.024</td>
</tr>
<tr>
<td>H-45</td>
<td>NT</td>
<td>NT</td>
<td>0.012</td>
</tr>
<tr>
<td>Hantaan (76-118)</td>
<td>- ‡</td>
<td>NT</td>
<td>NT</td>
</tr>
</tbody>
</table>

* The neutralizing activity was expressed as the amount of MAb (µg) necessary to reduce the number of foci (FRNT) or absorbance (NT-ELISA) by 80% using different concentrations of human complement (C).
† NT, Not tested.
‡ -, Negative.

MAb characterization

Thin-layer IEF revealed identical isoelectric patterns of the MAbs 1C9, 1E2, 2E8 and 2F10. The main band had an isoelectric point of 9.2. Typical microheterogeneity caused by variations in glycosylation of each clone was seen (data not shown). All four MAbs were found to be of the IgG1 λ subclass. None of the MAbs were reactive in immunoblot, suggesting reactivity with non-linear epitopes not recognized under denaturing conditions.

Epitope mapping

The epitope specificities of the human MAbs were determined by a competitive assay. The results indicated that MAbs 1C9, 1E2, 2E8 and 2F10 recognized identical or totally overlapping epitopes. Analogous results were obtained when epitope recognition was evaluated by an additivity ELISA (data not shown). When the bank vole MAb 4G2, reactive with the neutralizing G2a epitope, was included in the assays it was shown that all the human MAbs completely inhibited its binding. It was also shown that the MAb 4G2 inhibited the human MAbs, although to a lesser degree (Table 2).

Immunofluorescence patterns of viral antigen in acetone-fixed cells

The human MAbs gave identical immunofluorescence patterns in PUU virus-infected, acetone-fixed Vero E6 cells (Fig. 2a). The smooth diffuse staining pattern was found to be very similar to that obtained by the anti-G2 specific bank vole MAbs 4G2 and 5B7 (Fig. 2b and c).

Reactivity of MAbs against 11 hantavirus strains

Unconcentrated supernatants containing MAbs were titrated by IFA using 11 strains in the Hantavirus genus. The MAbs reacted with all eight PUU virus-like strains, but not with Hantaan, Seoul or Prospect Hill viruses which represent three other serotypes of hantaviruses. Minor differences in endpoint titres against the PUU virus-like strains were seen (Table 1).

Neutralizing activity of MAbs

The MAbs were tested for their neutralizing activities against PUU virus (strain 83-223L). All four MAbs had neutralizing reciprocal titres ranging from 8 to 32 when examined by FRNT as unconcentrated supernatants without complement. When human or guinea-pig complement (3.85%) was included in the NT-ELISA, the neutralization titres of MAb 1C9 were elevated 64- and 128-fold, respectively.

To estimate the neutralizing activity of MAb 1C9 in more detail, seven PUU virus-like strains, isolated in different parts of Europe, were examined in FRNT and/or NT-ELISA. All seven strains were neutralized completely by MAB 1C9, and the dose required to reduce the number of virus foci by 80% ranged from 12 to 24 ng (Table 3). The distinct increase of the neutralizing activity by supplement of human complement was clearly shown (Table 3).

The neutralizing activity of MAb 1C9 was further compared to the neutralizing activity of the immune serum from the spleen donor. The dose-response curves of equal amounts of IgG shown in Fig. 3 revealed that the neutralizing efficiency of MAb 1C9 was approximately 100-fold higher than the polyclonal human immune serum.

Growth characteristics and antibody secretion of monoclonal LCLs compared to human × mouse–human triomas

For stable and improved antibody secretion, three of the monoclonal LCLs, 1C9, 2E8 and 2F10, were fused with the non-secreting human × mouse heterohybridoma SPAM-8. A number (5 × 10⁶) of each LCL were fused at
clones and their corresponding LCL (1C9). The initial concentration used for initial screening. Altogether cells from 38 wells were counted and placed in culture flasks at an initial concentration of $1 \times 10^5$ cells/ml. Supernatants were harvested at days 4, 8, 12, 16 and 21 and examined in a G2-specific ELISA. The squares represent the mean absorbances of duplicate samples in the ELISA at different intervals (days) of cell culturing.

Fig. 3. Dose–response curves for virus neutralization. PUU virus (83-223L) was neutralized by human MAb 1C9 (○) or a high titre human NE convalescent serum (NT titre of 1:640) (▲) in the presence of 10% human complement.

Fig. 4. Comparison of the antibody production of different trioma clones and their corresponding LCL (1C9). The initial concentration of cells was $1 \times 10^5$ cells/ml. Cell supernatants were harvested at days 4, 8, 12, 16 and 21 and examined in a G2-specific ELISA. The squares represent the mean absorbances of duplicate samples in the ELISA at different intervals (days) of cell culturing.

Discussion

In a recent report, we described the generation of bank vole envelope glycoproteins expressed neutralizing domains (Lundkvist & Niklasson, 1992). MAbs of rodent origin are, however, recognized as non-self by the human immune system, and allergic reactions are therefore a significant risk in connection with their therapeutic use in man. Several investigations have shown a number of side effects such as fever, rashes, vomiting, urticaria, bronchospasm, tachycardia and dyspnoea (Borrebaeck, 1989). In addition, the efficiency of immunotherapy is often severely reduced owing to the anti-Ig response evoked in the patients receiving the treatment (Chatenoud, 1986).

The asparagine-linked carbohydrate sequences of human antibodies are more compatible with Fc receptors on human effector cells, as compared to carbohydrate sequences on rodent antibodies (Borrebaeck, 1989). An additional advantage is that a different repertoire of antibody specificities can be obtained with human MAbs which would allow for new and more finely tuned specificities compared to the rodent MAb repertoire (Borrebaeck, 1989). These facts prompted us to generate glycoprotein-specific human MAbs in order to obtain neutralizing reagents with a therapeutic potential.

Many different strategies for production of human MAbs have been employed since the first reports in 1977 (Rosén et al., 1977; Steinitz et al., 1977) and during recent years many promising human MAbs against different infectious agents have been produced (James & Bell, 1987). However, our novel approach using murine MAbs for indirect binding of the viral glycoproteins to magnetic beads followed by preselection of specific B lymphocytes prior to immortalization was shown to be a more convenient and time-saving technique compared to more traditional methods. Several advantages of this technique are: (i) time-consuming screening and cloning work was significantly reduced; (ii) the risk for overgrowth by non-producing or irrelevant cells during the initial phase was minimized; (iii) preselection by magnetic beads enriched clones secreting antibodies with high affinity (Ossendorp et al., 1989). The access to immune spleen B cells in this study enabled us to select from a vast pool of B cells compared to the limited number of suitable B cells available from peripheral blood lymphocytes (Lum et al., 1990). We believe that this new technique will also be valuable in the generation of human MAbs in other systems. In line with this, we have recently used the same method for production of an
Therefore, only MAb 1C9 was further characterized for NT assays, suggesting that the neutralizing epitope is isolated. The results showed no significant variation in neutralizing activity against the different PUU virus-like isolates. The identical subclass, isoelectric point, overlapping epitope recognition and neutralizing activity indicated the neutralization domain is immunodominant both in bank voles and in humans (Lundkvist & Niklasson 1992). However, the absence of cross-reactivities among rabbit anti-idiotypic antibodies generated against these two MAb indicated that they have a different fine specificity within the same neutralizing domain (Å. Lundkvist, C. Scholander & B. Niklasson, unpublished results). We therefore believe that neutralizing MAbs of the human antibody repertoire will be most valuable tools for future analyses of neutralizing epitopes and for vaccine design studies.

No specific treatment is available for NE patients at present. An antiviral drug, ribavirin, has been tried in a severe form of HFRS with some positive effect (Huggins et al., 1991). Use of immune plasma is presently being evaluated in at least two Russian studies on acutely ill NE patients, but no results are yet available. If immune plasma proves to be effective, human neutralizing MAbs would be a natural form of second generation therapy.

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References


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