Production of Human Monoclonal Antibody to X31 Influenza Virus Nucleoprotein

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SUMMARY

In vitro stimulation of human peripheral blood mononuclear cells with X31 influenza virus antigen has been used to enrich for specific anti-X31 antibody-producing cells. Following Epstein–Barr virus transformation of these stimulated cells, a cell line which produces human antibody to X31 virus was derived and subsequently cloned. The cloned cells secrete an IgG 1 k antibody which is directed against the nucleoprotein of A type influenza virus. Culture supernatants contain 10 to 20 µg/ml of specific antibody which is now used as a standard for the ELISA assay used in our laboratory to detect antibodies to influenza virus.

The production of human monoclonal antibodies in vitro would provide useful tools for the study of human immune responses to specified antigens, as well as giving valuable standard reagents for routine assay systems. One method which has been used in attempts to produce human monoclonal antibodies is the infection of antibody-producing B lymphocytes with the Epstein–Barr (EB) virus. This virus is a human herpesvirus which only infects B lymphocytes (Pattengale et al., 1973), transforming them into lymphoblastoid cell lines with the capacity for indefinite growth in culture (Pope et al., 1968). These transformed cells synthesize and secrete immunoglobulin (Rosen et al., 1977), and recently this fact has been exploited to produce cell lines which secrete specific antibodies (Steinitz et al., 1977, 1980; Kozbor et al., 1979; Kozbor & Roder, 1981). Cloning of these cell lines has led to the production in vitro of human monoclonal antibodies (Kozbor & Roder, 1981; Steinitz & Tamir, 1982). Several methods have been used to enrich for specific antibody-producing cells either before or after EB virus transformation. These include positive selection by rosetting with antigen-coated red blood cells (Steinitz et al., 1979, 1980; Kozbor et al., 1979), or negative selection by removal of surface immunoglobulin-positive B cells after allowing antigen binding followed by capping to occur (Kozbor & Roder, 1981). In the present study we have used a method of antigen stimulation of peripheral blood mononuclear cells (PBM) to produce a specific in vitro antibody response to influenza virus (X31) (Callard, 1979) in an attempt to enrich for anti-influenza antibody-producing B cells prior to EB virus transformation.

Blood was obtained from normal donors who were seropositive for anti-influenza virus antibodies, and whose lymphocytes were known to produce specific antibody when cultured with X31 antigen. The method used for X31 antigen stimulation has been described previously (Callard, 1979). Briefly, PBM at a concentration of $2 \times 10^6$ per ml were cultured in RPMI 1640 medium containing 2 g/l NaHCO$_3$, 25 mM-HEPES, 10$^{-5}$ M-hydrocortisone and 10% horse serum. Purified, infectious influenza virus strain X31 (kindly supplied by Dr J. Skehel, MRC, Mill Hill, London, U.K.) was added at a final concentration of 5 µg/ml. PBM were either infected with EB virus on day 0 or cultured with X31 antigen for 3 or 6 days before EB virus infection. Cells to be infected with EB virus were pelleted and resuspended at a concentration of $10^7$/ml in the supernatant culture medium of the B 95-8 cell line (Miller et al., 1972) which had been filtered through a 0.45 µm Millipore filter. The cells were incubated at 37 °C for 1 h, then pelleted and resuspended at the required concentration. The EB virus-infected cells, as well as control X31-stimulated, uninfected cells, were cultured at a concentration of $10^6$/ml in 0.2 ml aliquots of RPMI 1640 containing 2 mM-glutamine, 100 IU/ml penicillin, 100 IU/ml streptomycin, and 20% foetal calf serum in microtitre plates. Cyclosporin A (1 µg/ml) was added to the medium in some experiments in order to inhibit the activity of EB virus-specific cytotoxic
Table 1. *Antibody production in cultures following stimulation of PBM with X31 antigen*

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<tr>
<th>Expt. number</th>
<th>Days of antigen stimulation</th>
<th>Wells producing antibody (%)</th>
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<tr>
<td></td>
<td></td>
<td>EBV-transformed</td>
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<tr>
<td>1</td>
<td>0</td>
<td>2</td>
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<td></td>
<td>3</td>
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* Whole infectious X31 virus was kindly supplied by Dr J. Skehel.

T cells within the culture (Crawford, 1981). After a 1 month culture period, supernatant medium from all culture wells showing proliferating foci of cells, and from the uninfected control cultures (which did not contain proliferating foci of cells) was tested for specific anti-X31 antibody production by an enzyme-linked immunoabsorbent assay (ELISA) (Zanders *et al.*, 1981). Table 1 compares the number of culture wells in which anti-X31 antibody was produced following X31 antigen stimulation for 0, 3 or 6 days prior to EB virus transformation. In two experiments, only 2% and 0% of culture wells containing EB virus-infected, non-antigen stimulated PBM yielded supernatant medium containing specific antibody. Those cultures containing cells which had been stimulated for 3 days with X31 antigen gave 54% and 58% of supernatants which contained anti-X31 antibody; stimulation for 6 days gave 100% of antibody-containing supernatants in both experiments. Supernatants from uninfected cultures containing PBM which had been stimulated with X31 antigen for 0, 3 and 6 days, and treated in an identical way to the EB virus-infected cultures, contained no detectable specific antibody. These experiments indicate that *in vitro* stimulation of PBM with X31 influenza virus antigen leads to an enrichment of specific antibody-producing cells as shown by the increased percentage of culture wells containing EB virus-transformed cells which produce specific antibody after X31 antigen stimulation of PBM (Table 1). Cells from culture wells producing antibody were expanded and one was cloned three times by limiting dilution to give a monoclonal antibody-producing cell line (C10) which has been studied in detail. Expansion of cells from the uncloned antibody-producing cultures invariably leads to loss of antibody production after 6 to 12 weeks. This finding confirms the findings of other workers (Kozbor & Roder, 1981) and stresses the necessity for early cloning procedures.

Cytospin preparations of C10 cells were fixed in cold methanol and stained with mouse monoclonal antibodies to human immunoglobulin heavy and light chains (Lowe *et al.*, 1981), followed by a second layer of fluorescein-conjugated sheep antibody to mouse immunoglobulin. Around 90% of cells showed bright membrane fluorescence with antibodies to human IgG, IgG1 and light chain. No staining was detected with antibodies to human IgM, IgD (Naiem *et al.*, 1981), IgG2, IgG3, IgG4, or light chain. (These antibodies were kindly provided by Dr N. Ling, Birmingham, U.K.; Dr D. Mason, Oxford, U.K. and Dr M. Cooper, Birmingham, Ala., U.S.A.) Very few IgG class human monoclonal antibodies have been derived from EB virus-transformed cell lines, a fact which probably reflects the apparent preference of EB virus to infect IgM-bearing B cells (Rosen *et al.*, 1977). The quantity of specific anti-X31 antibody in the supernatant medium from cloned C10 cultures which had been left undisturbed for 5 days was measured by the ELISA technique using a standard serum of known antibody titre (Zanders *et al.*, 1981). The supernatants were found to contain 10 to 20 μg/ml of specific antibody. This quantity of monoclonal antibody produced compares favourably with that of other reported human monoclonal antibodies as well as that produced by the rodent hybridoma systems.

The specificity of the antibody was determined by immunoprecipitation with [*35S]*methionine-labelled extracts of influenza virus-infected chick embryo fibroblasts (CEF). Cells labelled from 4 to 6 h post-infection were lysed with 1% Nonidet P40 (NP40) and 1% sodium deoxycholate, clarified by low-speed centrifugation, and the resulting cytoplasmic extracts preadsorbed by incubation with 10% heat-inactivated *Staphylococcus aureus* Cowan I (Staph A) for 30 min at 20 °C.
Immunoprecipitation with an extract of X31-infected cells (Fig. 1a) showed that the C10 antibody binds to the virus nucleoprotein (NP). Immune precipitates prepared from C10 culture supernatants and cytoplasmic extracts of chick embryo fibroblasts infected with either an A-type influenza virus (A/Eq/Miami/63-H3N8; A/Swine New Jersey/8/76-H1N1; A/Brazil/11/78-H1N1; A/FPV (Rostock)/34-H7N1), or a B-type virus (B/Hong Kong/8/73) were run on SDS–polyacrylamide gels beside the matched extract (Fig. 1b). The bands indicate that C10 is specific for the nucleoprotein of type A influenza virus.

The cloned C10 cell line has now been growing in our laboratory for 12 months, during which time the antibody level in the supernatant medium has remained stable. The culture supernatant is now used as a standard in our ELISA assay. In indirect immunofluorescence tests, the antibody stains cells infected with A-type but not B-type virus. This may be of use for distinguishing between type A and type B viral infections.

Although it is not yet clear what proportion of specific antibody-producing cultures can be readily cloned to obtain stable antibody-producing lines, we feel that the method of antigen stimulation of PBM prior to EB virus immortalization described here is a useful way of enriching for specific antibody-producing cells.

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