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SPECIAL ARTICLE

The use of monoclonal antibodies in virology

Antibodies have been used for the last five decades in the laboratory diagnosis of a wide range of diseases caused by viruses and in detailed investigations of virus structure. However, immunological and serological assays have always had problems of interpretation, reproducibility and standardization, resulting partly from the unavoidable heterogeneity of the antibodies in the test. When a mouse, for example, is immunized with a virus, the animal may easily recognise 10–20 different antigenic determinants. As many as five distinct antibodies can be produced by the mouse against each determinant and these will often differ from antibodies made by another mouse against the same antigenic determinant. The method of lymphocyte fusion and the subsequent generation of monoclonal antibodies (Kohler & Milstein, 1975; Kohler, 1980) overcomes these limitations.

As a general procedure, spleens of previously immunized mice are fused to a myeloma cell line which grows in tissue culture. Mice may be immunized with partially purified preparations of virus with satisfactory results since clones reacting with contaminating proteins are screened out later in the technique. The mixture of fused and unfused cells is plated out in a highly selective medium The unfused spleen cells die in the tissue culture medium and also the unfused myeloma cells die. The latter death results from a technical trick of obtaining myeloma cells defective in the enzyme hypoxanthine phosphoribosyl transferase (HGPRT). The selective tissue culture medium is supplemented with hypoxanthine, aminopterin and thymidine (HAT medium). In this medium the main pathway of cellular DNA synthesis is blocked by aminopterin while, in addition, the alternative rescue pathway cannot be carried out in the myeloma cells because of the myeloma cell deficiency in HGPRT. In contrast, hybrids between the myeloma and spleen cells survive, the spleen cells supplying functional HGPRT enzyme and myeloma cells supplying ability to grow in tissue culture. Such hybridomas may produce up to 100 μg of antibody/ml in culture and 10 mg/ml in the ascitic fluid of a tumour-bearing mouse. The hybrid cell lines grow continuously in culture and can be frozen in liquid nitrogen. Thus, the production of a particular homogeneous antibody can be immortalized. Usually only one in 2×10^5 spleen cells may actually form a viable hybrid with a myeloma cell. Since each mouse spleen contains around 10^8 nucleated cells, 300–500 hybrids can be generated from each spleen. If the mixture of spleen and myeloma cells is plated out at 5×10^4 myeloma cells per well in a 96 well microtitre dish, approximately one third of the wells will contain a growing hybrid between 2–3 weeks after fusion. At this time the supernatants of the clones are screened for specific antibody, usually by a sensitive radioimmune assay or ELISA test. It cannot be overemphasized that the sensitivity and specificity of this assay is particularly crucial to the whole hybridoma technique. Clones producing

antibody can be recloned and then frozen away. To avoid the earlier complication of hybridomas continuing to make both myeloma and spleen immunoglobulins, non-immunoglobulin-producing myeloma cell lines have been isolated and are now widely used (Shulman, Wilde & Kohler, 1978, reviewed by Kennett, McKearn & Bechtol, 1980).

A typical experiment to produce hybridomas is described in some detail by Tedder, Yao & Anderson (1982) in the previous issue of the *Journal*. The authors have obtained a number of cell lines secreting antibodies to rubella virus without the use of complex laminar flow cabinets. These and others emphasize how small variations in the technique, such as the use of different batches of PEG of varying molecular weight for the fusion process or the use of feeder cell layers can enhance or decrease the number of antibody positive clones. Tedder, Yao & Anderson (1982) noted that early cloning of hybrid parents secreting antibody yielded only a few antibody positive clones, whereas a higher proportion of rubella antibody positive clones arose if the parent hybrid culture had been allowed a longer period of growth prior to cloning. It would appear from this paper that the technique, perhaps unlike genetic engineering technology, can be exploited rapidly and easily in laboratories without extensive biochemical or tissue culture facilities. Also, at present most laboratories which have produced monoclones against different viruses are willing to share ascitic fluids although not usually the hybridoma cell lines themselves.

Already some important advances in knowledge concerning 'molecular epidemiology' and structure of a number of viruses have been made using monoclonal antibodies. The use of monoclones as highly specific reagents in studies of virus epidemiology is becoming more widespread and two papers in the *Journal* from the PHLS Laboratory in Colindale describe this application to influenza A virus (Pereira & Chakraverty, 1982; Chakraverty, Cunningham & Pereira, 1982). In addition, several groups of investigators are currently using monoclones to search for unambiguous evidence of antigenic variation among diverse groups of human viruses where variation had not been demonstrated before or alternatively where precise quantitation of this variation was lacking. Thus, recently, antigenic variants of the Edmonston strain of measles virus have been detected using immunoselection techniques and monoclonal antibodies (Birrer *et al.* 1981). In this approach a cloned virus is grown in the presence of an excess of neutralizing monoclonal antibody, and any 'breakthrough' viruses analysed. It is inferred that variants arise spontaneously in tissue culture and are isolated due to the selection pressure of antibody. Monoclonal antibodies were obtained in high titre from ascitic fluid after implantation of hybridoma cells into the peritoneal cavity of pristane treated mice. With each monoclone 1 ml of ascitic fluid was capable of neutralizing 200000 PFU virus. Selection in the presence of a monoclonal antibody yielded three antigenic mutants of measles virus which were not neutralized by the monoclonal antibody, although the growth of parental virus could be completely suppressed. The frequency at which mutants arose was approximately 10^{-4}. The variants seemed to differ from wild type virus in at least one antigen determinant on the HA and were genetically stable mutants because *in vitro* passage resulted in no change in the pattern of neutralization. An important conclusion is that

measles virus should now be re-examined for antigenic differences between vaccine strains and various wild type isolates. Furthermore, since the measles virus genome is subject to mutation in the region coding for the HA antigen it should be possible to determine if antigenic variants have any role in the persistence of measles-like viruses *in vivo* as observed in subacute sclerosing panencephalitis. Giraudon & Wild (1981) have independently prepared a number of monoclonal antibodies to the L, HA and NP polypeptides of the Halle strain of measles virus and used them as immunological probes in measles virus and asociated diseases. The authors were able to detect antigenic variants of measles virus but a larger library of clones will be required to completely differentiate viruses from different origins.

Antigenic variants of influenza virus (Gerhard & Webster, 1978; Lubeck, Schulman & Palese, 1980) and rabies virus (Wiktor & Koprowski, 1980) can be selected with relative ease in the laboratory using monoclonal antibodies. In a recent study reported in the *Journal*, antigenic mutants of influenza A/Texas/77 (H3N2) virus were selected after cultivation of virus in the presence of excess monoclonal antibody to the HA antigen (Natali, Oxford & Schild, 1981). These mutants failed to react with certain human antisera, particularly from children, although the same sera reacted with the parental A/Texas/77 virus. An important conclusion was that a change in all four antigenic determinants or epitopes of influenza haemagglutinin was not a prerequisite for the selection of a variant of epidemiological significance. Earlier studies (Yewdell, Webster & Gerhard, 1979) had indicated that antigenic variants to a single monoclonal antibody might occur with a frequency of 1 in 100000 virions. Since at least four independent antigenic sites are known to occur on the influenza HA, the frequency of occurrence of variants with changes on all sites would be extremely low (1 in 10^{24}). Therefore it was difficult to envisage how any new antigenic variants could arise in nature, escape neutralization and spread in the community. The data from the two more recent studies (Natali, Oxford & Schild, 1981; Haaheim, 1980) explains this paradox. Certain human sera possess a more limited anti-influenza virus antibody repertoire (compared to experimental animal sera) which is restricted to one epitope. This potentially allows such antigenic variants to escape neutralization and spread in the community. Furthermore, since a higher proportion of children's sera, compared to adult sera, failed to react with the antigenic variant, an interesting conclusion was that new influenza antigenic variants may be generated preferentially in children, and thereafter spread to adults.

Lubeck, Schulman & Palese (1980) observed a wide range of frequencies at which antigenic variants were found in influenza A and B virus populations following selection with monoclonal antibodies using the techniques described above. Influenza B/Lee/40 virus antigenic variants were found at frequencies of 10^{-7} or less, whereas antigenic variants were obtained with A/PR/8/34 (H1N1) virus with a relatively high frequency ($10^{-4.1}$). Several possible explanations of these findings can be considered including differences in viability or growth potential of different variants in the cell culture system, differences in mutational frequencies of sequences coding for different antigenic sites, and differences in avidity of monoclonal antibodies used in the selection procedures.

More recently Webster and Berton (1981) have studied antigenic drift in a wide range of influenza B viruses using a collection of 12 monoclones. The antigenic determinants on the HA could be subdivided into three partially overlapping groups and antigenic drift occurred in each of the epitopes defined by the 12 different monoclonal antibodies. Two observations were of particular interest. Firstly the study showed conclusively that at least two antigenically different influenza B variants could co-circulate in a community at the same time. Secondly the B/HK/8/73 virus was more closely related antigenically to A/Bon/43 and B/Sweden/49 than to recent influenza B isolates. This latter observation is worthy of more detailed study. The frequency of antigenic variation in the HA molecule of B/HK/8/73 was less than 1 in 10^8, being 100 to 1000-fold below that found with influenza A viruses as described above. This suggests that the HA of influenza B viruses is less variable than that of influenza A virus. It is unlikely that the mutation frequency in the RNA coding for influenza B virus HA is less than that in influenza A viruses. Other possible explanations are that the influenza B virus populations contain a high frequency of phenotypically mixed particles or that the majority of mutations are lethal, implying that there might be more functional restrictions on the confirmation of the influenza B HA molecule.

Influenza antigenic variants selected *in vitro* using monoclonal antibodies are also useful for detailed investigations of the molecular basis for antigenic variation. The complete amino-acid sequence of several influenza HAs has now been determined and also the tertiary structure of the HA protein has been determined by X-ray crystallography (Wilson, Skehel & Wiley, 1981). Air, Laver & Webster (1980) have described several monoclonal variants of A/Hong Kong/68 (H3N2) virus which show single sequence changes in or close to peptide 17. Most of these variants showed a change in the proline residue at position 143 in the HA1 polypeptide. In these monoclonal variants the proline at position 143 changed to serine, threonine, leucine or histidine. Thus, this particular region of the HA molecule can accommodate many changes in sequence which are responsible for changes in the antigenicity of the HA. It is not clear at present if amino-acid changes occurred in the actual antigenic site recognized by the monoclonal antibody or if they were located elsewhere and induced conformational changes which altered the antigenic site. At least a beginning has been made towards correlating changes in amino-acid sequences with changes in antigenic properties. Such detailed knowledge will be required for any future attempts to produce by genetic cloning in bacterial or animal cells, synthetic vaccines consisting of antigenic portions of the HA.

An important study of antigenic variation in rabies virus has been reported by Wiktor & Koprowski (1980). CVS-11, a standard challenge rabies virus was grown in the presence of a concentration of monoclonal antibody able to neutralize approximately 10^5 infectious units of virus. Antigenic variants in single glycoprotein epitopes were present in the cloned rabies seed at a frequency of $10^{-4.3}$ to 10^{-5} and the variants probably represent single point glycoprotein mutants of the CVS virus. Moreover, the study showed that virus isolated from seven fatal cases of human rabies represented antigenic variants. Rabies virus appears to have a similar

potential as influenza to undergo antigenic variation under suitable experimental conditions in the laboratory. But what is the relevance to field studies? Post-exposure treatment of rabies is not 100% effective and failure to protect exposed persons has been attributed in the past either to a low potency of the vaccine or to a delay in beginning treatment. It is now apparent that a third factor could be antigenic differences among strains of rabies virus. The authors noted that vaccine prepared from antigenic variants did not protect mice against challenge with either parental or another variant virus. Since relatively large numbers of street virus variants exist in different parts of the world the current methods of selection and testing rabies strains for vaccine production may have to be modified. Meanwhile the libraries of monoclones against rabies are proving useful as immunological probes for studies of the intercontinental spread of particular rabies variants.

Russell *et al.* (1981) have characterized monoclonal antibodies against the major structural and non-structural components of human adenoviruses type 5. At least five different epitopes were found to be associated with the hexon antigen and preliminary data suggests that complex inter-relationships between the hexons of different serotypes may exist. In addition, further subdivision of the various adenovirus types can probably now be achieved using these monclones. The anti-hexon hybridomas could prove to be valuable probes in diagnostic virology enabling rapid typing of virus and perhaps finer discrimination of virus types in association with particular disease syndromes. However, the anti-hexon hybridomas failed to neutralize virus infectivity and were not active in CF tests and therefore at present the use of these reagents is limited to immune-fluorescence tests.

Studies of monoclones against mouse mammary tumour viruses have led to proposals for a general mechanism of virus neutralization (Massey & Schochetman, 1981). These authors demonstrated a unique site involved in virus neutralization and predicted that changes in epitopes adjacent to the cell receptor binding site would affect antibody-mediated virus neutralization. According to this hypothesis, influenza mutants selected using monoclones as described above would have an additional alteration in an epitope near the binding site. Epitopes which have a low frequency of change and are highly conserved may be of most interest. For example, if a receptor binding site is conserved among all variants then a good candidate vaccine strain would be a virus which elicits the most marked immunological response to this epitope.

Monoclonal antibodies are being extensively used to dissect apart the antigenic structure of poliovirus (Icenogle *et al.* 1981; Ferguson *et al.* 1981), influenza (Van Wyke, Bean & Webster, 1981) and murine leukaemia viruses (Lostrom *et al.* 1979) revealing new antigenic relationships between viruses and new structural relationships between proteins of a single virus. A neutralizing monoclonal antibody against type 1 poliovirus reacted not only with native virions but also with naturally occurring RNA-free protein shells (70S) and with 14S assembly subunits previously thought to lack native virus antigenic sites. This latter observation may have important implications for the development of picornavirus

sub-unit vaccines since it is possible that the non-infective 14S subunits may be active in stimulating the formation of neutralizing antibodies (Icenogle *et al.* 1981) as has been shown for 12S subunits of foot and mouth disease virus.

Several clones of hybridomas secreting antibody to alpha and beta interferons have now been obtained (Secher & Burke, 1980, Hochkeppel, Menge & Collins, 1981). Monoclonal anti-beta interferon antibodies neutralize the antiviral activity of human beta interferon but not that of human alpha interferon. Such antibody may be useful for the purification of cloned interferon.

Finally, the limitations of monoclonal antibodies as reagents and probes should not be forgotten. They cannot always form the complex lattices necessary for precipitation in immunodiffusion tests, immunoelectrophoresis or routine agar diffusion and some classes or subclasses of monoclonal antibodies do not fix complement. In addition, for front line virological diagnosis some of the monoclones may be too specific, recognizing only a particular virus variant or serotype, when what may be required, in the first instance, is an antibody reacting widely with all viruses of the group. In fact it may be necessary to use a cocktail of several monoclonal antibodies reacting with different epitopes for diagnostic purposes. In this regard the use of monoclonal antibody in the antibody capture assay for detection of rubella virus specific IgM described in the *Journal* (Tedder, Yao & Anderson, 1982) is of particular interest since over-specificity is not relevant. The monoclonal antibody is needed only to recognize the homologous virus epitope. A biased repertoire of antibody specificities is another potential complication in the interpretation of results using hybridomas since the Balb/c mice often used for these experiments may possess a limited antibody repertoire for the particular virus antigen. Finally, of course, monoclonal antibodies are not strictly monospecific, because an individual paratope (or antibodies produced by a hybridoma and expressing a single set of immunoglobulin variable region genes) may bind with different avidities to a panel of related virus antigens, or more importantly a single hybridoma antibody may cross-react with a viral protein and a cell protein which have an epitypic relationship (reviewed by Yewdell & Gerhard, 1981). Nevertheless, at present the potential problems of interpretation are more than counterbalanced by the very significant amounts of new data already produced and also by the future scientific potential of this new technology. Papers already published in the *Journal* have emphasized the successful application of hybridoma technology for studies on molecular variation, epidemiology and for precise clinical diagnostics. Future areas of research will undoubtedly include detailed investigations of the structure and immunology of rubella, measles, polio and related enteroviruses, rapid identification of viruses in clinical samples, virus interactions with cells and the immunology of important parasitic infections such as malaria (Cox, 1981).

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REFERENCES

AIR, G. M., LAVER, W. G. & WEBSTER, R. G. (1980). Towards a universal influenza vaccine. In *New Developments with Human and Veterinary Vaccines* (ed. A. Miztahi, I. Hertman, M. A. Klingberg and A. Kohn). New York: Alan R. Liss.

BIRRER, M. J., UDEM, S., NATHENSON, S. & BLOOM, B. R. (1981). Antigenic variants of measles virus. *Nature* **293**, 67–69.

CHAKRAVERTY, P., CUNNINGHAM, P. & PEREIRA, M. S. (1982). The return of the historic influenza A H1N1 virus and its impact on the population of the United Kingdom. *Journal of Hygiene* **89**. (In the Press.)

COX, F. E. G. (1981). Hybridoma technology identifies protective malaria antigens. *Nature* **294**, 612.

FERGUSON, M., SCHILD, G. C., MINOR, P. D., YATES, P. J. & SPITZ, M. (1981). A hybridoma cell line secreting antibody to poliovirus type 3 D antigen: detection in virus harvest of two D antigen populations. *Journal of General Virology* **54**, 437–442.

GERHARD, W. & WEBSTER, R. G. (1978). Selection and characterisation of antigenic variants of A/PR/8/34 (H1N1) influenza virus with monoclonal antibodies. *Journal of Experimental Medicine* **148**, 383–392.

GIRAUDON, P. & WILD, T. F. (1981). Differentiation of measles virus strains and a strain of Canine Distemper Virus by monoclonal antibodies. *Journal of General Virology* **57**, 179—183.

HAAHEIM, L. R. (1980). Haemagglutination-inhibition antibodies in human sera to an antigenic mutant of influenza A/Texas/1/77 (H3N2) virus obtained *in vitro*. *Acta Pathologica Scandinavica* **88**, 351–356.

HOCHKEPPEL, H. K., MENGE, U. & COLLINS, J. (1981). Monoclonal antibodies against human fibroblast interferon. *Nature* **291**, 500–501.

ICENOGLE, J., GILBERT, S. F., GRIEVES, J., ANDEREGG, J. & RUECKERT, R. (1981). A neutralising monoclonal antibody against poliovirus and its reaction with related antigens. *Virology* **115**, 211–215.

KENNETT, R. H., MCKEARN, T. J. & BECHTOL, K. B. (Eds.) (1980). *Monoclonal Antibodies*. New York: Plenum.

KOHLER, G. & MILSTEIN, C. (1975). Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature* **256**, 495–497.

KOHLER, G. (1980). Hybridoma techniques. *Cold Spring Harbour Laboratory Manual*.

LOSTROM, M. E., STONE, M. R., TAM, M., BURNETTE, W. N., PINTER, A. & NOWINSKI, R. C. (1979). Monoclonal antibodies against murine leukaemia viruses; identification of six antigenic determinants on the p15(F) and gp70 envelope proteins. *Virology* **98**, 336–350.

LUBECK, M. D., SCHULMAN, J. L. & PALESE, P. (1980). Antigenic variants of influenza viruses: marked differences in the frequencies of variants selected with different monoclonal antibodies. *Virology* **102**, 458–462.

MASSEY, R. J. & SCHOCHETMAN, G. (1981). Topographical analysis of viral epitopes using monoclonal antibodies: mechanism of virus neutralisation. *Virology* **115**, 20–32.

NATALI, A., OXFORD, J. S. & SCHILD, G. C. (1981). Frequency of naturally occurring antibody to influenza virus antigenic mutants selected *in vitro* with monoclonal antibody. *Journal of Hygiene* **87**, 185–191.

PEREIRA, M. S. & CHAKRAVERTY, P. (1982). Influenza in the United Kingdom 1977–81. *Journal of Hygiene* **88**, 501–512.

RUSSELL, W. C., PATEL, G., PRECIOUS, B., SHARP, I., & GARDNER, P. S. (1981). Monoclonal antibodies against adenovirus type 5: preparation and preliminary classification. *Journal of General Virology* **56**, 393–408.

SECHER, D. S. & BURKE, D. C. (1980). A monoclonal antibody for large scale purification of human leukocyte interferon. *Nature* **285**, 446–450.

SHULMAN, M., WILDE, C. D. & KOHLER, G. (1978). A better cell line for making hybridomas secreting specific antibodies. *Nature* **276**, 269–270.

TEDDER, R. S., YAO, J. L. & ANDERSON, M. J. (1982). The production of monoclonal antibodies

to rubella haemagglutinin and their use in antibody capture assays for rubella-specific IgM. *Journal of Hygiene* **88**, 335–350.

VAN WYKE, K. L., BEAN, W. J. & WEBSTER, R. G. (1981). Monoclonal antibodies to the influenza A virus nucleoprotein affecting RNA transcription. *Journal of Virology* **39**, 313–317.

YEWDELL, J. W. & GERHARD, W. (1981). Antigenic characterisation of viruses by monoclonal antibodies. *Annual Review of Microbiology* **35**, 185–206.

YEWDELL, J. W., WEBSTER, R. G. & GERHARD, W. U. (1979). Antigenic variation in three distinct determinants of an influenza type A haemagglutinin molecule. *Nature* **279**, 246–248.

WEBSTER, R. G. & BERTON, M. T. (1981). Analysis of antigenic drift in the haemagglutinin molecule of influenza B virus with monoclonal antibodies. *Journal of General Virology* **54**, 243–251.

WIKTOR, T. J. & KOPROWSKI, H. (1980). Antigenic variants of rabies virus. *Journal of Experimental Medicine* **152**, 99–112.

WILSON, I. A., SKEHEL, J. J. & WILEY, D. C. (1981). The haemagglutinin membrane glycoprotein of influenza virus: structure at 3 Å resolution. *Nature* **289**, 366–373.