The production of monoclonal antibodies to rubella haemagglutinin and their use in antibody-capture assays for rubella-specific IgM

By R. S. TEDDER, J. L. YAO

Department of Virology, Middlesex Hospital Medical School,
Riding House Street, London W1P 7PP

AND M. J. ANDERSON

Department of Medical Microbiology, King's College Hospital Medical School,
Denmark Hill, London SE5 8RX

SUMMARY

Mice were immunized by three intraperitoneal and one intravenous injection of rubella haemagglutinin. Splenocytes from these mice were fused with the cells of a syngeneic myeloma cell line, and following culture for various periods of time, single-cell clones were derived by the technique of limiting dilution.

A total of 139 clones were derived from 13 parent hybrid cultures. To date, four of these cloned cultures have been propagated as ascitic tumours in mice. The preparation of IgG from ascitic fluid and labelling of this antibody with ^{125}I is described. Results indicate that the use of labelled monoclonal antibodies as indicator reagents in solid-phase IgM antibody capture assays for the detection of rubella-specific IgM results in enhanced performance of these tests.

INTRODUCTION

The principle of diagnosing viral infection by the detection of virus-specific IgM antibody in the patient's serum is now well accepted. The earliest methods relied upon the physical separation of IgM class antibodies from those of the IgG class, and the subsequent determination of antiviral activity in these fractions. The best known of these techniques concern the detection of IgM antibody specific for rubella virus. Density gradient ultracentrifugation and column chromatography have been widely used to achieve separation of antibody classes, and the haemagglutination-inhibition (HI) test employed to detect specific antibody in the fractionated serum. Similar technology has proved difficult to apply to the diagnosis of infection with viruses lacking haemagglutinin since the other widely used system for detecting antiviral antibody, the complement fixation test, does not permit the detection of IgM-class antiviral antibody.

However, more recently, the antigenic differences of the antibody classes have

been exploited; antisera with specificity for IgM and IgG antibodies have been labelled, initially with fluorescein isothiocyanate, and in recent years with radio-isotopes and colour-producing enzymes. The first generation of immunoassays employing these labelled anti-globulin preparations involved reacting the test serum with viral antigen on a solid phase such as an infected cell or plastic surface. Bound IgM (or IgG) antibodies were then detected by subsequent binding of labelled class-specific anti-globulin.

Of late, a new generation of tests has been devised, where the anti-globulin antibodies themselves are used to 'fractionate' the test serum (Diament & Pepys, 1978). These 'antibody capture' assays involve adsorbing the anti-IgM antibody to a solid phase, such as a polystyrene bead, and then binding IgM from the test serum to this solid phase. The specificity of the IgM is determined by reacting the captured antibody with viral antigen. Bound viral antigens are detected either by intrinsic labelling of the antigen (Schmidt, Deimling & Flehmig, 1980) or by the application of a labelled antiviral antibody. Several assays of this type have now been described for the detection of IgM specific for a number of viruses including hepatitis A virus (Duermeyer, Wielaard & van der Veen, 1979), hepatitis B virus (Gerlich & Luer, 1979) and rubella virus (Mortimer et al. 1981b).

Antibody capture assays offer potentially more sensitive systems for the detection of low levels of antiviral IgM than do the first generation of immuno-assays by virtue of the pentameric valency of IgM. However, to realize this poten-tial, a labelled antibody preparation capable of detecting low concentrations of virus is required. To date, this has often involved raising hyperimmune animal antisera. These must then be carefully evaluated for unwanted reactivities which should be removed whenever possible.

The description by Kohler & Milstein (1975) of a method of obtaining pure antibody preparations with a single specificity raised the exciting possibility of replacing hyperimmune animal sera with these purpose-made reagents. This technique involves the isolation of individual antibody-secreting B cells fused with the immortal cells of a myeloma, to yield hybrid daughter cell populations of identical cells producing a single monoclonal antibody.

We describe here the application of this method to the production of clones of hybrid cells secreting antibody to the haemagglutinin (HA) of rubella virus, and the use of these antibodies labelled with a radioisotope in an IgM antibody-capture radioimmunoassay (MACRIA) for the detection of rubella-specific IgM (Mortimer et al. 1981b).

MATERIALS AND METHODS

Antigens

RK 13 cells were infected with the Judith strain of rubella virus. Four days after inoculation the cultures were subjected to cycles of freezing at −70 °C and thawing at 37 °C. This preparation was further disrupted by sonication, clarified by centrifugation at 2000 g for 15 min and stored at −70 °C. This preparation is referred to hereinafter as RUBa.

Rubella HA (PHLSHA) was obtained from the Division of Microbiological

Reagents and Quality Control of the Public Health Laboratory Service, and had a titre of 128 HA units. Further HA antigen and a control antigen prepared from uninfected BHK cells was purchased from Wellcome Reagents Ltd. This HA antigen (Wellcome HA) had a titre of 1024 while the control preparation had no HA activity.

Animals

Balb/c mice were obtained from Bantin and Kingman Ltd and bred in the School of Pathology, Middlesex Hospital Medical School, for use throughout this work.

Immunisation. Mice were injected intraperitoneally (I.P.) with 250 μl RUBa mixed with an equal volume of Freund's complete adjuvant. Seventy and 240 days later a further dose of 100 μl RUBa mixed with an equal volume of 20 mM phosphate-buffered saline pH 7·6 (PBS) was given I.P. Two weeks after the third dose, tail vein blood was assayed for rubella-specific IgG. Forty days later 250 μl PHLSHA mixed with an equal volume of PBS were injected intravenously (I.V.). Three days later, mice were killed by cervical dislocation and the spleens removed aseptically.

Production of ascitic fluid. Female Balb/c mice aged 12–14 weeks were treated with 0·5 ml Pristane (Sigma Ltd) injected I.P. One to three weeks later between 5×10^6 and 2×10^7 viable cloned hybrid cells suspended in 1 ml RPMI medium were injected I.P. Mice usually developed ascitic fluid two to three weeks after inoculation. When the swelling was easily visible fluid was removed by aspiration from the peritoneal cavity with a hypodermic needle.

Tissue culture

All cell-culture procedures were performed on the open bench in the vicinity of a Bunsen burner flame. Tissue culture microplates with 96 wells (T 96 plates) and tissue culture plates with 24 wells (T 24 plates) were purchased from Costar Ltd. Tissue culture flasks with growth areas of 25 cm^2 and 80 cm^2 (Nunc) were purchased from Flow Laboratories Ltd. These culture vessels together with disposable pipettes (Volac Ltd) were used throughout this study. Cell cultures were incubated at 37 °C in an atmosphere of 5 % CO_2 in air at almost 100 % humidity in a GA2 CO_2 incubator (LEEC Ltd). Cells in culture were examined under phase contrast illumination while determinations of cell viability and concentration were made by counting cells suspended in 0·5 % trypan blue in PBS in improved Neubauer chambers.

Three tissue culture media were used; complete medium (CM) consisted of RPMI medium supplemented with 5 mM Hepes buffer and 2 mM L-glutamine (Flow Laboratories Ltd) together with 0·05 mM 2-mercaptoethanol (Koch-Light Ltd.) and 20 % v/v fetal calf serum (FCS; SeraLab Ltd). The following antimicrobial agents were added to all media: Fungizone (2·5 μg per ml), penicillin (10^2 units per ml) and streptomycin (100 μg per ml).

Selective medium (HAT) was complete medium supplemented with 10^{-4} M hypoxanthine, 4×10^{-7} M azoguanine and $1·6 \times 10^{-5}$ M thymidine (all supplied by Sigma Ltd).

Rescue medium (HT) was the same as selective medium but without azoguanine.

Preparation of feeder cell layers. Two cell types were used to assist the growth of hybrid cells; peritoneal macrophages were obtained by lavage of freshly sacrificed mice with 5 ml ice-cold RPMI. Cells harvested from a single animal were pelleted by centrifugation at $50\,g$ for 5 min at 4 °C and resuspended in 10 ml complete medium. Such a preparation was sufficient for either a single T96 tissue culture plate (100 μl containing approximately 10^5 cells per well) or a single T24 tissue culture plate (400 μl containing approximately 4×10^5 cells per well).

Thymocytes were obtained by teasing apart in ice-cold RPMI the thymus which had been aseptically removed from freshly killed mice aged between three and four weeks. The cells were pelleted by centrifugation at $50\,g$ for 5 min at 4 °C, washed once in RPMI and resuspended in complete medium to a concentration of $5-10 \times 10^5$ cells per ml. For use in T24 plates 5×10^5 cells per well were used and 3×10^6 cells per flask in 25 cm^2 tissue culture bottles.

Myeloma cells. JK-Ag 8653 cells from Balb/c mice were grown in CM in 80 cm^2 tissue culture flasks. The cells were maintained at a concentration of between 2×10^5 and 2×10^6 cells per ml. For use in fusion, cells in the log phase of growth were washed twice in ice-cold RPMI without serum and resuspended to a concentration of 10^7 cells per ml in the same medium.

Spleen cells. The spleen was removed aseptically from immunized mice three days after they had received their final i.v. inoculation. The organs were disrupted with forceps in ice-cold CM and the cell suspension allowed to stand for 5 min, during which time the debris settled. The supernatant was aspirated from the debris and the splenocytes in the supernatant washed twice in RPMI medium. Finally the spleen cells were resuspended to a concentration of 10^7 cells per ml in RPMI medium without serum.

Cell fusion. Immune spleen cells were mixed with myeloma cells in the ratio of five splenocytes to one myeloma cell in a 50 ml conical-bottomed centrifuge tube, and pelleted by centrifugation at $50\,g$ for 5 min. The supernatant was removed by aspiration to leave the cell pellet dry, and the tube placed in a water bath at 37 °C. A 50% solution of polyethylene glycol (BDH Ltd, GLC grade, 1540) in RPMI was pre-warmed to 37 °C and 500 μl cells added dropwise to the tube while the cell suspension was gently stirred with a pipette. After gentle mixing for 1 min, 1 ml RMPI at 37 °C was added dropwise during the course of 1 min while the cell mixture was constantly stirred. Further RMPI at 37 °C was added slowly so that the volume was doubled every minute until a final volume of 25 ml was obtained.

The fused cells were pelleted by centrifugation at $50\,g$ for 5 min and gently resuspended to a concentration of 3.5×10^6 viable cells per ml in HAT medium.

Culture of fused cells. The fused cells were cultured initially on feeder layers of peritoneal macrophages in HAT which had been inoculated into the tissue culture wells 24 h previously. The fused cells were dispensed onto these feeder macrophages in 100 μl aliquots (3.5×10^5 cells/well) and incubated at 37 °C in an atmosphere of 5% CO_2 in air. Every four days 100 μl medium was withdrawn from each well and replaced with a similar volume of HAT medium. After 14 days culture, the replacement medium was changed to HT medium.

Fourteen days after fusion, when the fused cells were growing well, they were transferred from the wells of T96 microplates to the larger wells of T24 plates. The wells of these plates had been prepared with macrophage feeder layers as described above and the medium had been changed to HT medium. All of the cells from each well of the T96 plate were aspirated into a sterile pipette and transferred to separate wells in the fresh T24 plates. Care was taken to avoid cross-contamination of separate cultures. The cells were maintained in these larger wells by the replacement of 50% of the medium volume with CM in the manner previously described.

After a further period of growth in these tissue culture plates, the cells from selected wells were transferred to 25 cm² tissue culture flasks. These flasks contained feeder layers of mouse thymocytes, and supported the growth of the hybrid cells for between two and seven days, fresh medium being added every few days. When rapid cell growth was established, the hybrid cells were stored under liquid nitrogen in CM containing 10% dimethylsulphoxide (DMSO) and 50% FCS, pending their further use.

Cloning. This was carried out by limiting dilution; cells from wells whose supernatant medium gave positive results in the assay for rubella-specific IgG (described below) were suspended in CM at a concentration of 70 cells per ml. One hundred microlitres of cell suspension were dispensed into each well of a T96 tissue culture plate containing macrophage feeder cells. Seven days later 100 μl fresh medium were added to each well. During this period the cultures were examined daily with an inverted microscope and after 7 days those wells with only a single clone of cells visible were marked. When these cells had grown sufficiently to cover between one-third and one-half of the well, 100 μl medium was withdrawn from each of these marked wells and assayed for murine anti-rubella IgG.

Clones producing this antibody were transferred to the larger wells of T24 plates as described above and then passed into 25 cm² tissue culture flasks containing feeder cell layers of thymocytes. When these cells were dividing rapidly they were transferred to 80 cm² tissue culture flasks without feeder cells. The hybrid cells were maintained in log phase growth, with fresh medium being introduced every few days. During this period of growth the cells were examined regularly for viability, and the cell concentration held between 5×10^5 and 2×10^6 cells per ml. A portion of these cells were injected I.P. into mice for the production of ascites while the remainder were stored under liquid nitrogen.

Preparation of monoclonal antibodies

Recovery of IgG antibody from ascitic fluid. Immunoglobulin was purified from ascitic fluid by chromatography of the fluid on 10 ml columns of DE52 (Whatman Ltd). One ml volumes of ascitic fluid were dialysed overnight at 4 °C against 10 mM phosphate buffer, pH 8·0 and applied to the top of the columns which had been pre-equilibrated with the same buffer. After allowing proteins to adsorb to the cellulose for 30 min, they were eluted; twenty ml of 10 mM phosphate buffer were applied to the column and any eluted protein collected. Thirty ml of 30 mM phosphate buffer were then applied and any further eluted protein collected. Finally, 30 ml of 60 mM phosphate buffer were applied to elute any remaining

protein. Each of these protein preparations was assayed separately for rubella HI antibody and the fraction containing the majority of antibody activity brought to a protein concentration of 1 mg per ml, based on $E_{1\,cm}^{1\%} = 14$ for murine IgG.

Radioiodination of IgG antibody. Monoclonal antibodies were labelled with ^{125}I by the method of Salacinski *et al.* (1979) using iodogen. The antibody preparation was dialysed overnight against 0·1 M borate-buffered saline, pH 8·0 and the protein labelled in the ratio 120 μg protein to 1 mCi ^{125}I in tubes coated with 5 μg iodogen (Wariner–Pierce Ltd). Free iodine was separated from that bound to the globulin by fractionation on columns (7 cm × 0·64 cm^2) of Sephadex G-25. The protein-rich fractions were stored at 4 °C in the elution buffer, Tris-buffered saline, pH 7·6, containing 0·1 % sodium azide with 5 % bovine serum albumin (BSA).

Assay of rubella HI antibody. This was performed by the method described by Pattison & Mace (1973). Briefly, 8 HA units of PHLSHA were incubated with serial twofold dilutions of the fluid under test in Lucite microtitre plates for 2 h at room temperature. Pigeon erythrocytes were added and the plate incubated at 4 °C for 1 h. The plates were then allowed to warm to room temperature and the patterns examined. The HI titre of the preparation was taken as the reciprocal of the dilution at which complete inhibition of haemagglutination was observed.

The FCS used throughout this study contained non-specific inhibitors of rubella HA; however, these were present in titres of less than 50. Thus it was possible to test tissue culture supernatants containing 20 % FCS for HI activity at dilutions of 1 in 10 and greater without any necessity for prior treatment to remove these inhibitors.

Solid-phase radioimmunoassay for rubella-specific murine IgG. The production of anti-rubella antibody in immunized mice, by hybrid cell cultures, and by clones was monitored using a solid-phase radioimmunoassay (SPRIA) for rubella-specific IgG based on that described by Kangro, Pattison & Heath (1978) for the detection of human rubella-specific IgG. The methods used in the assay of murine virus-specific IgG will be described elsewhere (Anderson, manuscript in preparation). Briefly, anti-mouse IgG, purchased from Gibco Europe Ltd, was purified by affinity chromatography on mouse IgG coupled to CNBr-activated Sepharose 6 B (Pharmacia Ltd) and labelled with ^{125}I, using the iodogen method, in the ratio 50 μg protein to 1 mCi ^{125}I.

The solid-phase antigen was prepared as described by Kangro, Pattison & Heath (1978) by desiccating Wellcome HA or control antigen onto the wells of flexible polyvinyl microtitre plates and fixing with 10 % formalin. Remaining protein-binding sites were blocked by overnight incubation with PBS containing 0·2 % gelatin at 4 °C. The plates were stored dry at −70 °C for up to one month.

Fifty μl of either mouse serum diluted 1 in 100, or hybrid culture supernatant diluted 1 in 2 or 1 in 10 in PBS containing 5 % FCS were incubated in duplicate antigen-coated wells for 1 h at 37 °C. The solid phase was then washed three times with PBS containing 0·05 % Tween 20 (PBST) using the Dynatech Microwash system, and reacted for 1 h at 37 °C with 50 μl radio-iodinated anti-mouse IgG, diluted in PBST to contain 30000 counts per minute (c.p.m.) per 50 μl. The

radiolabel was then aspirated from the wells and the solid phase washed 10 times. Finally the wells were clipped from the plate and the bound radioactivity counted in a 16 channel gammacounter (NE 1600, Nuclear Enterprises Ltd).

Hybrid culture supernatants were tested initially for affinity for HA antigen; the supernatants containing strongly positive activity were then tested for affinity for uninfected BHK cell control, antigen.

Investigation of the properties of the monoclonal antibodies

The monoclonal antibodies produced in ascites were first examined to determine the degree of similarity between their antigenic specificities and then their performance in M-antibody capture assays for rubella-specific IgM.

Cross-competition experiments. Flat-bottomed polystyrene wells (Removawell; Dynatech Ltd) were coated with rubella HA antigen by incubating 100 μl PHLSHA antigen diluted 1 in 100 in 0·02 M Tris buffer, pH 7·6, for three days at room temperature. After washing with Tris buffer, the wells were filled with 0·5 % BSA in Tris buffer (TRIS-BSA) and held for 1 h at room temperature. The wells were stored moist and sealed at 4 °C. Immediately before use the wells were aspirated to dryness.

^{125}I labels prepared from individual clones were diluted in TRIS-BSA to contain the same level of radioactivity. A series of half-log$_{10}$ dilutions of unlabelled IgG from each clone were prepared in TRIS-BSA to contain protein in the range 100 μg to 30 ng per ml. Fifty microlitres each of labelled IgG and an unlabelled IgG preparation were added to duplicate wells in such a way that each label competed in turn with itself and each of the other three antibody preparations for the HA antigen on the surface of the well. The wells were incubated overnight at room temperature, washed, and the bound radioactivity measured.

MACRIA for rubella-specific IgM. The assay was performed essentially as described by Mortimer *et al.* (1981 *b*). Simply, 6·4 mm diameter polystyrene beads were coated with anti-human IgM. Immediately prior to use the beads were incubated for 3 h at room temperature in PBS containing 1 % BSA. Sera containing a range of concentrations of rubella-specific IgM from 100 to 0·3 units per ml were diluted 1 in 40 in PBST. Two hundred μl of diluted serum were reacted with the anti-IgM coated beads for 3 h at 37 °C. After washing, the beads were incubated overnight at 4 °C in PHLSA antigen diluted 1 in 10 in PBST. The beads were again washed and incubated with one of each of the four monoclonal antibodies, or the mixture of equal parts of all four, diluted to contain 50000 c.p.m. per 200 μl, or a ^{125}I-labelled rabbit anti-rubella IgG preparation diluted to contain 100000 c.p.m. per 200 μl. The diluent for each of these labels consisted of PBS with 0·2 % Tween 20 and 30 % heat-inactivated homologous sera without detectable anti-rubella antibody. The radiolabels were incubated with the beads for 3 h at 37 °C, after which the beads were again washed in PBST and the radioactivity bound measured.

Fig. 1. Solid-phase radioimmunoassay (SPRIA) of rubella-specific IgG in supernatant media of hybrid parent microcultures. Control medium from cultures of macrophage feeder cells bound the ^{125}I-labelled anti-mouse IgG to give between 300 and 500 counts per 100 s. Hybrid parent cultures A to H inclusive (▼), were cloned by limiting dilution immediately following this assay. Hybrid parent cultures I to P inclusive (■), were cloned by limiting dilution following 11 days culture in T 24 tissue culture plates.

RESULTS

Production of cloned hybrids secreting anti-rubella antibody

Ten days after fusion the supernatant fluids from the hybrid microcultures were tested for rubella-specific IgG. Ninety-two of the 96 wells contained cells which were secreting anti-rubella IgG; between 2 and 13 times as much radiolabel was bound to wells incubated with supernatant media from these cultures as was bound to wells incubated with medium from macrophage feeder cells alone (Fig. 1). The antibodies were titrated in the SPRIA by making serial dilutions in control medium. The specificity of the antibodies was confirmed by the failure of the supernatants to react at a 1 in 2 dilution with control BHK cell antigen.

The cells from each of these 92 wells giving positive results in the SPRIA were transferred to the larger wells of T24 plates to allow expansion of the cultures. Twenty-two of these cultures, producing high levels of antibody, were marked for further investigation. After a period of growth, the remaining 70 cultures were stored under liquid nitrogen

Of the 22 cultures, the 8 most active cultures (A–H, Fig. 1) were immediately cloned by limiting dilution. Eleven days later, those wells with only single clones of cells visible with the inverted microscope were tested for the production of rubella-specific IgG (Fig. 2). The supernatant media from the majority of these clones did not react significantly at a 1 in 5 dilution with Wellcome HA. In the case of the clones derived from two of the parent cultures, none of the 22 single clones from parent D or of the 23 from parent H was found to secrete significant amounts of antibody. The remaining 6 parent cultures yielded a total of 20 clones secreting
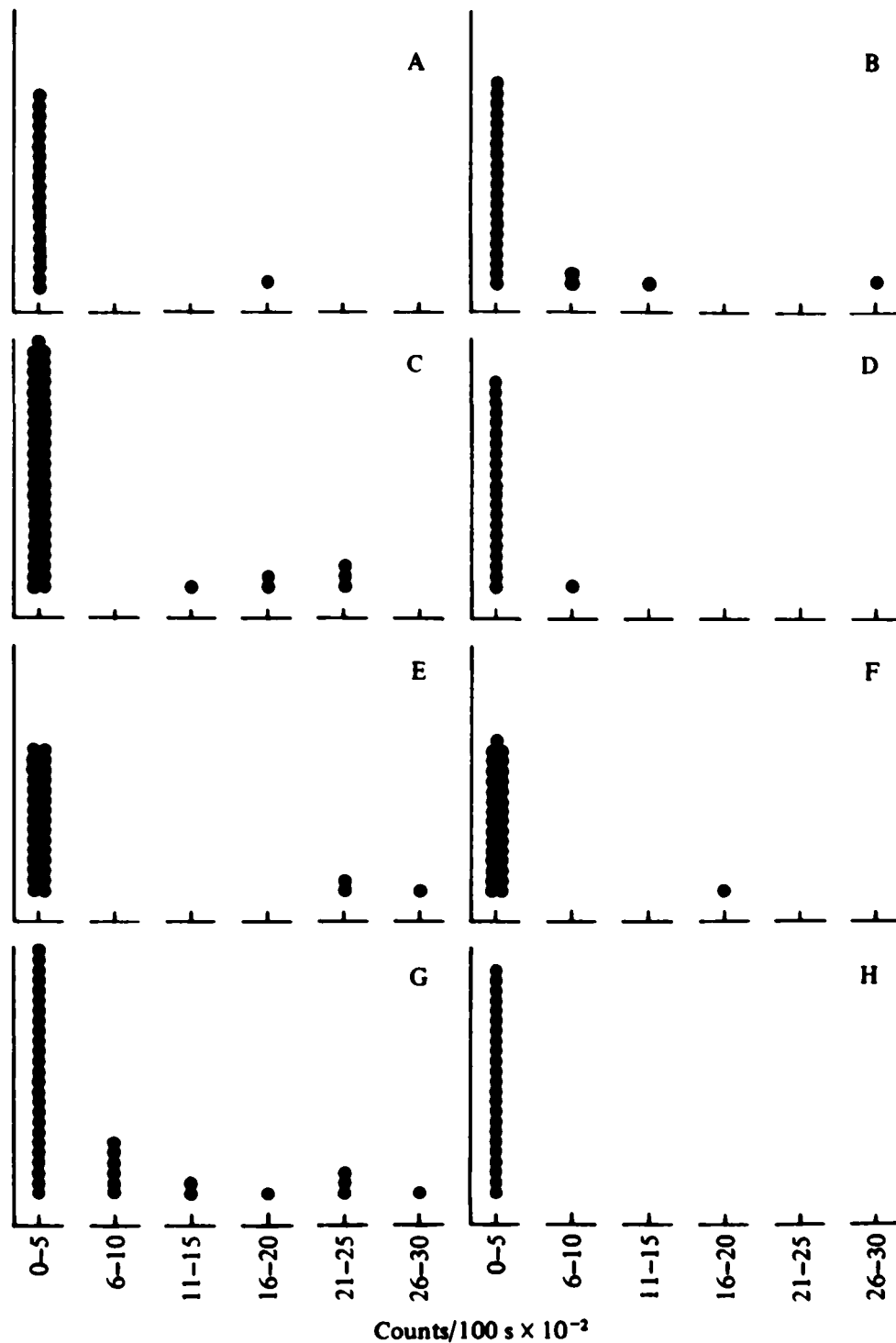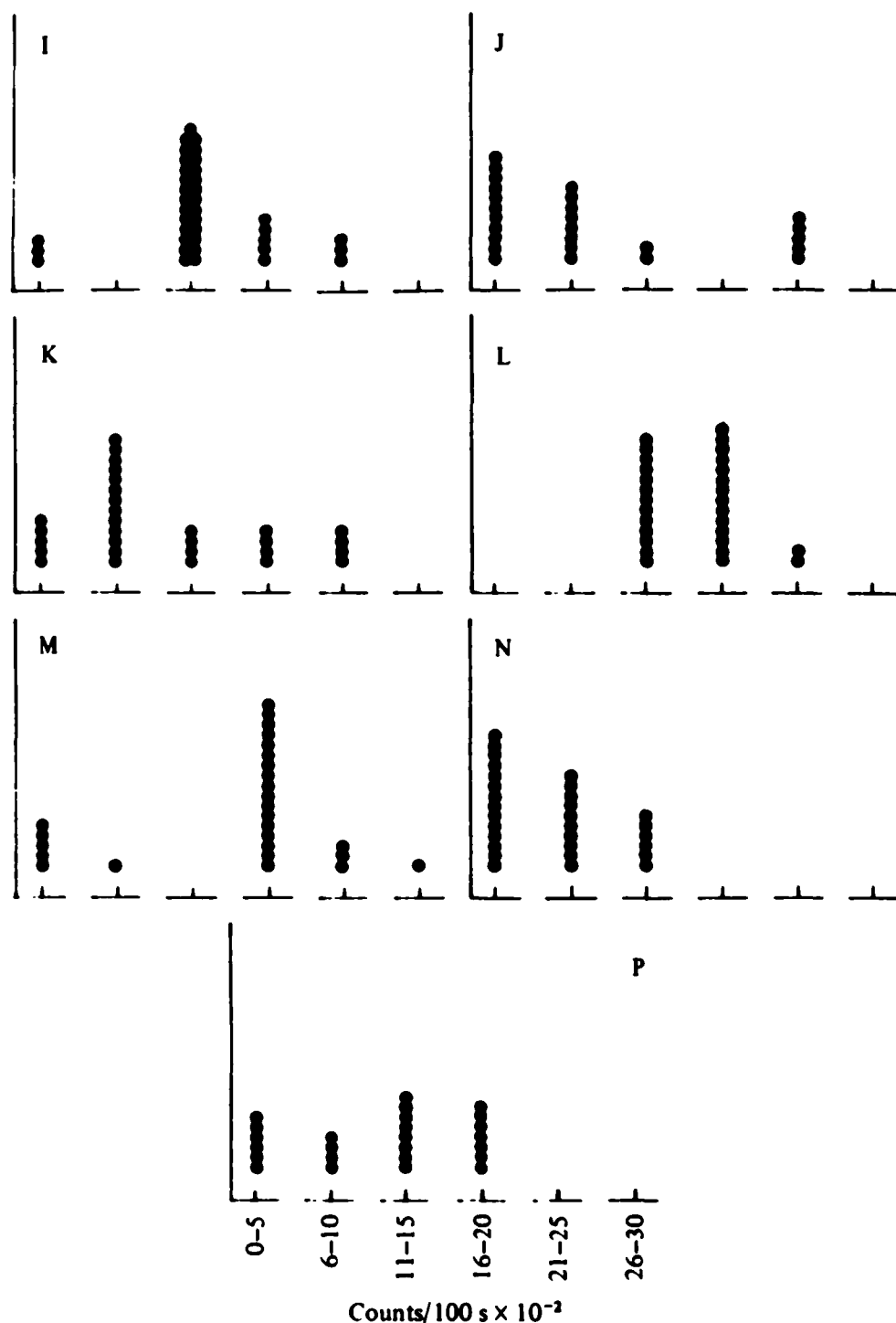
Fig. 2. Screening the clones arising from hybrid parent cultures A to H by SPRIA for the production of rubella-specific IgG. Control medium from macrophage feeder cell layers bound between 300 and 500 counts per 100 s. Each point represents the radioisotope bound by supernatant medium from one T 96 tissue culture well containing a single colony of cells 11 days after cloning by limiting dilution.

significant amounts of antibody. Fourteen of these clones producing the highest levels of antibody were selected for propagation, and the remainder discarded.

Three weeks after fusion, the other 16 hybrid cultures growing in T 24 plates were examined for the production of antibody reactive in both the SPRIA and HI tests. The seven cultures (I–P, Fig. 1) producing the highest titres of antibody detected in both tests were cloned by limiting dilution. The supernatant media from wells

12-2

Fig. 3. Screening the clones arising from parent hybrid cultures I to P by SPRIA for the production of rubella-specific IgG. Control medium from macrophage feeder cell cultures bound between 300 and 500 counts per 100 s. Each point represents the ^{125}I bound by supernatant medium from one T 96 tissue culture well containing a single colony of cells 11 days after cloning by limiting dilution.

containing only single colonies of cells were tested for rubella-specific IgG (Fig. 3). In contrast to the clones of younger hybrid parent cultures, all of these older parents gave rise to antibody-producing clones. A total of 125 clones secreting significant amounts of rubella antibody was derived from the seven parents. The proportion of clones secreting anti-rubella antibody ranged from 20 % (parent N) to 100 % (parent L).

From these two cloning procedures 139 antibody-secreting clones were derived

from 13 parent cultures; six of these parents had been selected by their production of antibody detected in the SPRIA alone while the older parents were selected by SPRIA together with HI. Each of the antibody-secreting clones was allowed to grow in T24 plates and was then transferred to 25 cm² tissue culture flasks. This expansion step was most successful when thymocytes were used as feeder cells in the flasks at a concentration of 5×10^6 cells per ml. Rapid cell growth was encouraged by maintaining the cloned cell concentration between 5×10^5 and 2×10^6 cells per ml. When rapid cell growth was established feeder cells were no longer necessary. From the 139 clones derived from the 13 parents, 14 producing antibody reactive in both tests were selected for propagation. To date four of these, MR1 to MR4, have been successfully passed into mice and propagated as ascitic tumours.

Yield and purification of ascitic fluid antibody. Mice carrying hybridomas as an ascitic tumour usually exhibited ascites 10–14 days after I.P.inoculation of tumour cells. As soon as the swelling was easily visible the ascites was aspirated every two to three days.

The total yield of ascitic fluid obtained from each mouse ranged between 1·0 ml and 25 ml. The outcome of I.P. injection appeared to be a growth characteristic of the individual clone; in mice where the tumours grew as a solid mass only small volumes of fluids were produced. However, it was in the fluid from such animals that the highest levels of IgG were found, exceeding in some cases 15 mg per ml.

Immunoglobin G was prepared from the ascitic fluid as described. In each case the bulk of the protein and HI antibody eluted as a single peak in either the 30 mM fraction (MR2 and MR3) or in the 60 mM fraction (MR1 and MR4). Yields of purified mouse IgG varied between 8 mg per mouse for MR2 and 50 mg per mouse for MR3.

Investigation of clone specificity. Mixtures of labelled and unlabelled immunoglobulin were incubated in wells coated with rubella HA antigen, and the ability of each immunoglobulin to inhibit the binding of label prepared either from the same or another clone was assessed. Figure 4 illustrates the inhibition of binding of radiolabelled MR4. Inhibition by unlabelled MR1 and MR3 is similar to that found with the homologous antibody MR4. Much less inhibition is found with MR2. Similar results were obtained using radiolabelled MR1 and MR3. However, the uptake of radiolabelled MR2 was unaffected by the presence of the other three monoclonal antibodies. Thus the four antibodies react with two epitopes of the HA antigen, the specificities of clones MR1, MR3 and MR4 being closely related.

The use of monoclonal anti-rubella antibody in the IgM antibody-capture assay

In spite of the similarities in specificity displayed by the three monoclonal antibodies MR1, MR3 and MR4, the performance of all four monoclonal antibodies, and a mixture of equal parts of all four, was examined in the rubella MACRIA. The reactivity in this assay of control sera containing known amounts of rubella-specific IgM ranging from 0·3 to 100 units per ml, and a pool of eight sera containing no such antibody, was determined using a radio-iodinated preparation of each of the four monoclonal antibodies and of the pool. The percentage of the
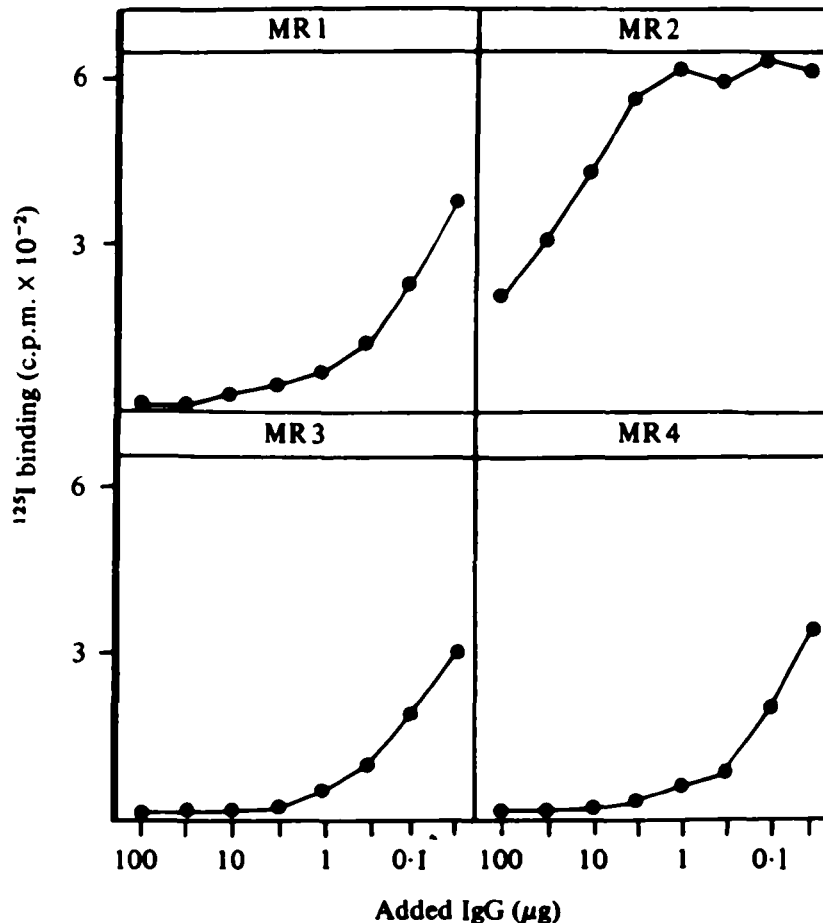
Fig. 4. The effect of addition of unlabelled anti-rubella IgG on the binding of ^{125}I-labelled MR 4 IgG to solid-phase rubella HA antigen. Purified IgG from each clone, MR 1 to MR 4 inclusive, was mixed separately in the amounts shown with ^{125}I-labelled MR 4 anti-rubella IgG and the mixture incubated overnight in wells coated with rubella HA antigen. The wells were subsequently washed and the binding of radiolabel measured and expressed in c.p.m.

available radiolabel bound by the control sera was related to the rubella-specific IgM content of these sera (Fig. 5).

In the presence of small amounts of rubella-specific IgM (0·3–3·0 units per ml) the binding to the solid phase of the labels produced from each of the four clones was very similar. However, with sera containing higher concentrations of anti-rubella IgM there was increasing divergence between the proportions bound. Labels prepared from MR 1, MR 3 and MR 4 gave very similar dose-response curves. In contrast, the label prepared from MR 2 appeared relatively insensitive to increasing levels of rubella-specific IgM and no distinction could be made between sera containing 30 and sera containing 100 units per ml of antibody. The radiolabel prepared from the mixture of all four monoclonal antibodies was influenced very little by the inclusion of MR 2, and gave a dose–response curve of the same form as those obtained with MR 1, MR 3 and MR 4.

The results obtained in a rubella MACRIA using a radiolabelled pool of the monoclones MR 1–MR 4 were compared with those obtained using a hyperimmune rabbit serum (Table 1). The percentage of the radiolabel bound was 8–26 times
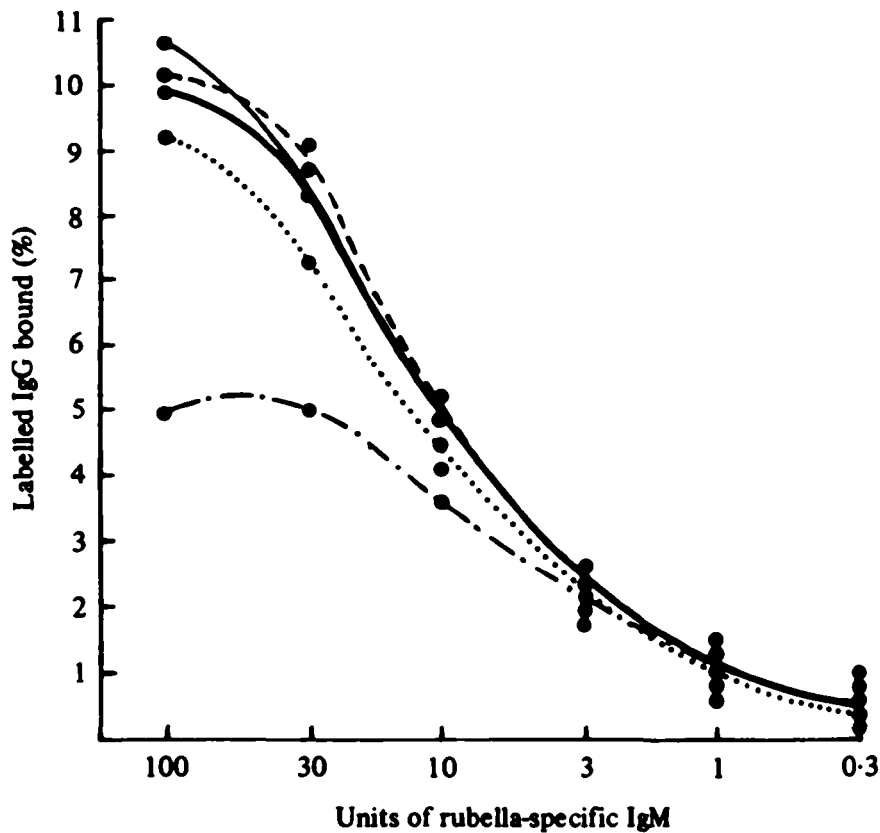
Fig. 5. The application of ^{125}I-labelled monoclonal antibodies in an IgM antibody-capture assay of rubella-specific IgM. Polystyrene beads coated with anti-IgM globulin were incubated with sera containing defined concentrations of rubella-specific IgM. Following reaction of the beads with rubella HA antigen, the percentage of available ^{125}I-labelled monoclonal antibody bound was determined; antibody derived from MR1 (●——●), MR2 (●—·—●), MR3 (●···●), MR4 (●———●) and the radio-iodinated pool of equal parts of all four monoclonal antibodies (●——●). Beads reacted in the first stage with rubella antibody negative control sera bound 0·2 % of the available radiolabel from each preparation.

Table 1. *The use of a pool of monoclonal antibodies compared with hyperimmune rabbit serum globulins in the rubella MACRIA*

| | ^{125}I-labelled preparation | | | |
| Rubella-specific IgM (units) | Pooled monoclonal antibodies | | Hyperimmune rabbit serum | |
	% bound	P/N*	% bound	P/N
100	9·9	44·5	0·37	9·3
30	8·3	41·5	0·30	7·5
10	4·9	24·5	0·27	4·3
3	2·5	12·5	0·15	3·8
1	1·1	5·5	0·10	2·5
0·3	0·5	2·5	0·06	1·5
Nil	0·2	—	0·04	—

* Ratio between positive and negative control serum.

greater with the monoclonal pool than with the hyperimmune serum. Moreover the positive/negative (P/N) ratio was greater with the monoclonal pool throughout the range of IgM-containing standards tested.

DISCUSSION

The IgM antibody-capture assay for rubella-specific IgM is a sensitive test, capable of detecting IgM responses in recipients of rubella vaccine (Mortimer et al. 1981 b). However, a major difficulty of this test has been the preparation of a suitable labelled indicator antibody. For this reason we embarked upon the development of monoclonal anti-rubella antibodies, this new technology appearing to offer a secure supply of pure antibody.

Initially we feared that the lack of a laminar flow cabinet would be a considerable hindrance, but during the course of this work no infections have occurred in any of our cultures. The use of the solid-phase radioimmunoassay to detect rubella-specific antibody permitted rapid screening of hybrid culture supernatant media for antibody and greatly facilitated the detection of colonies secreting antibody following cloning by limiting dilution. In the later stages of growth hybrids were selected by determination of the HI titres of the supernatant media. Although it would have been possible to use this method throughout to select clones, the relative insensitivity of this assay may have required further expansion of clones prior to testing. It was possible to quantify the reactivity of antibody in the SPRIA by assay at a single dilution. Moreover, this assay was able to identify clones secreting antibody against antigens other than the HI. However, since the SPRIA detected only IgG antibody, the sole use of this in screening supernatant media will have resulted in the loss of antibody classes other than IgG.

Early cloning of hybrid parents secreting antibody yielded only a few antibody-positive clones. This may have been due to the cells having been seeded at too high a concentration immediately following fusion, resulting in a high multiplicity of clones arising from each hybrid micro-culture. In contrast, 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. This could be explained if the slower growing clones were lost as the cell cultures were expanded. Thus, cultures remaining HI and SPRIA positive after protracted culture could be expected to contain, at most, only a few rapidly dividing clones. Early cloning therefore favours the individual selection of most of the antibody-secreting clones, although for a given number of colonies arising from each parent the frequency of specific antibody-positive clones will be low. In order to obtain all the possible antibody-secreting clones present in young hybrid cultures it may be necessary to undertake extensive cloning. A less labour-intensive method is to allow the initial hybrid cultures to expand and grow in culture, thereby selecting for fast-growing cells. When these cultures are cloned they may yield almost pure-culture progeny, as was seen with parent L, where all the colonies seemed to be derived from the same antibody-secreting stock. A disadvantage in this method is the loss of slow-growing, antibody-producing clones.

Antibodies produced by clones from single cells have a very narrow range of specificity; this has been held by some authors to be overspecific (David *et al.* 1981). Small alterations in an antigen, such as are caused by single-point nucleotide deletions, may very significantly alter the avidity of a monoclonal antibody for the antigen (Gerhard *et al.* 1981). Were these antibodies to be employed in the detection of infectious agents from clinical material this overspecificity may indeed have a profound effect on the success of such a technique, since it is increasingly apparent that a single pathogen, homogeneous when investigated by conventional serology, may in fact comprise a range of variants (Webster & Berton, 1981). In order to recognize and detect all variants of a particular pathogen it may thus prove necessary to use mixtures of a number of monocolonal antibodies (Goodall *et al.* 1981).

In the IgM antibody-capture assay overspecificity is not relevant, since the antibody is required only to recognize the antigen against which it was raised. An obvious advantage of a monoclonal antibody in an assay of this type is the purity and specificity of the resulting label. To date it has proved impracticable to produce pure, specific anti-rubella antibody by the more conventional approach of affinity chromatography; this may have been due in part to the instability of the rubella antigens. As a result, labels used in antibody-capture assays have contained, in addition to the rubella-specific component of the IgG fraction, much redundant IgG, with the concomitant risk of false-positive reactions. These may be due to rheumatoid factor, anti-species antibody or reactions with antigens present in commercial preparations of rubella HA antigen which are themselves not rubella-specific. This last factor may be of particular significance in assays for IgM antibody where the antigen preparation used is of low purity, for example the faecal extracts used in assays for IgM antibody to hepatitis A virus. Thus the use of monoclonal antibody preparations in antibody-capture assays generally may be expected to result in assays which are not only more sensitive, but also of greater specificity.

The degree to which this potential will be realized remains to be determined. The value of a label prepared from hybridoma antibodies for use in the assay described by Mortimer *et al.* (1981*a*) is currently under assessment. Of particular interest will be the capacity of the test to detect rubella-specific IgM in recipients of rubella vaccine. There is a need for an especially sensitive IgM assay in investigating pregnant women inadvertantly vaccinated with rubella. It has been suggested that failure to detect an IgM response in vaccinees is indicative of prior immunity. If the vaccinee is a pregnant woman, the pre-existing antibody may further reduce the already low risk of fetal damage by the vaccine virus (Mortimer *et al.* 1981*a*). The most sensitive test that can be devised will then be valuable in identifying accurately those women who produced IgM, who were susceptible to infection and whose fetuses were at a higher risk.

We have described the production of monoclonal antibodies to rubella antigens and their use as radioisotope-labelled reagents in antibody-capture assays for rubella-specific IgM. These reagents have significantly enhanced the performance of the assay. Other applications of these antibodies, such as the characterization

of rubella antigens and the detection of rubella antigen in clinical material, remain to be explored. It is hoped this work may stimulate other workers to embark on the production, for both scientific and reagent use, of monoclonal antibodies to other agents which, like rubella, have proved difficult to purify.

REFERENCES

DAVID, G. S., PRESENT, W., MARTINIS, J., WANG, R., BARTHOLOMEW, R., DESMOND, W. & SEVIER, E. D. (1981). Monoclonal antibodies in the detection of hepatitis infection. *Medical Laboratory Sciences* **38**, 341–348.

DIAMENT, J. A. & PEPYS, J. (1978). Immunosorbent separation of IgG and IgM for the radio-immunoassay of specific antibodies. In *Affinity Chromatography* (ed. Hoffmann-Ostenhoffer *et al.*), pp. 229–231. Oxford: Pergamom Press.

DUERMEYER, W., WIELAARD, F. & VAN DER VEEN, J. (1979). A new principle for the detection of specific IgM antibodies applied to an ELISA for hepatitis A. *Journal of Medical Virology* **4**, 25–32.

GERHARD, W., YEWDELL, J., FRANKEL, M. E. & WEBSTER, R. (1981). Antigenic structure of influenza virus haemagglutinin defined by hybridoma antibodies. *Nature* **290**, 713–717.

GERLICH, W. H. & LUER, W. (1979). Selective detection of IgM antibody against core antigen of the hepatitis B virus by a modified enzyme immunoassay. *Journal of Medical Virology* **4**, 227–238.

GOODALL, A. H., MIESCHER, G., MEEK, F. M., JANOSSY, G. & THOMAS, H. C (1981). Monoclonal antibodies in a solid phase radiometric assay for HBsAg. *Medical Laboratory Sciences* **38**, 349–354.

KANGRO, H. O., PATTISON, J. R. & HEATH, R. B. (1978). The detection of rubella-specific IgM antibodies by radioimmunoassay. *British Journal of Experimental Pathology* **59**, 577–583.

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

MORTIMER, P. P., EDWARDS, J. M. B., PORTER, A. D., TEDDER, R. S., MACE, J. E. & HUTCHIN-SON, A. (1981*a*). Are many women immunized against rubella unnecessarily? *Journal of Hygiene* **87**, 131–138.

MORTIMER, P. P., TEDDER, R. S., HAMBLING, M. H., SHAFI, M. S., BURKHARDT, F. & SCHILT, U. (1981*b*). Antibody capture radioimmunoassay for anti-rubella IgM. *Journal of Hygiene* **86**, 139–153.

PATTISON, J. R. & MACE, J. E. (1973). Rubella screening tests. *Journal of Clinical Pathology* **26**, 161.

SALACINSKI, P., HOPE, J., McCLEAN, C., CLEMENT-JONES, V., SYKES, J., PRICE, J. & LOWRY, P. J. (1979). A new simple method which allows theoretical incorporation of radioiodine into proteins and peptides without damage. *Journal of Endocrinology* **81**, 131–137.

SCHMIDT, H., DEIMLING, U. VON & FLEHMIG, B. (1980). Detection of IgM antibodies to cytomegalovirus (CMV) using an enzyme-labelled antigen (ELA). *Journal of General Virology* **50**, 59–68.

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