Immunology, 1963, **6**, 439.

Antigenic Specificities Acquired from the Growth Medium by Cells in Tissue Culture

R. N. HAMBURGER,* D. A. PIOUS† AND S. E. MILLS

Biology Department, University of California, San Diego, La Jolla, California

(*Received* 15*th January* 1963)

Summary. Studies employing quantitative complement fixation have shown that HeLa and other mammalian cells grown in tissue culture adsorb serum protein components from the growth medium. The serum proteins are not completely removed by vigorous washing. Upon injection of extensively washed cells into rabbits the bound serum proteins give rise to specific antibodies detectable by gel diffusion and complement fixation. With horse serum in the growth medium one of the cell-bound components was identified as horse γ globulin. Evidence was obtained that specific antibodies to bound serum antigens can contribute to complement-dependent kill of the cells *in vitro*. These observations suggest one possible mechanism for the acquisition of antigenic relationships by diverse cell lines grown in tissue culture.

INTRODUCTION

Methods now available for the isolation and subculture of clones of animal cells in tissue culture make it possible to examine the properties of relatively homogeneous cell populations as distinct from the heterogeneous cell populations normally encountered *in vivo*. Certain constraints on such an analysis can, however, arise from the tissue-culture procedure itself. In the course of an antigenic analysis of cloned cell strains of murine and human origin it was observed that changes in culture conditions could introduce variation in the antigenic expression of the cells. An important source of variation was apparently contributed by the serum used in the growth medium.

It has previously been shown by other investigators that cells cultured *in vitro* can incorporate serum components of the growth medium. Gaillard (1953) observed the persistence of cat serum proteins in homogenates of rabbit ovarian fragments cultured in cat serum. Coombs, Daniel, Gurner and Kelus (1961) demonstrated the conversion of human HeLa and mouse 'L' cell populations from Forssman-negative to Forssman-positive by cultivation in sera containing Forssman antigen.

Serum, a standard component of the growth medium of most established cell lines, is essential to the establishment of primary cell strains and is known to combine with the surface of animal cells. If this union were firm enough to defy dissociation by the methods commonly employed to prepare cells for immunization and serological analysis, it could introduce complicating factors in the analysis, possibly by masking cellular antigens or by contributing additional specificities.

To examine these possibilities, a cloned HeLa cell line was grown in media containing

* Supported by Special Fellowship BT654 of the National Institute of Neurological Diseases and Blindness, U.S. Public Health Service.

† Post-doctoral Fellow, Division of General Medical Sciences, U.S. Public Health Service.

serum from different sources and an examination made of the antigenic properties of the ensuing populations. The present report describes some of the results obtained in this analysis.

MATERIALS AND METHODS

Media

The synthetic media employed were N16 (Puck, Cieciura and Robinson, 1958) and M199 (Morgan, Morton and Parker, 1950). Growth medium consisted of synthetic medium supplemented with 20 per cent (v/v) horse, rabbit or foetal calf serum. To obtain growth with rabbit serum it was necessary to heat the serum at 56° for 30 minutes.

Horse serum was obtained through the courtesy of the San Diego Zoological Society. Rabbit serum was collected at Champion Farms (El Cajon, California). Foetal calf serum was purchased from Microbiological Associates, Inc.

Buffered saline G (Puck *et al.*, 1958) was used for washing and cell dilutions. The concentrations of Ca^{++} and Mg^{++} in saline G are those required for the reactions of guinea-pig complement.

Cells

HeLa cells, obtained from Microbiological Associates (Bethesda, Maryland), were expanded in horse serum medium and transferred to medium containing heated rabbit serum or foetal calf serum as required. Stock suspensions were preserved at −70° in 10 per cent glycerol and 90 per cent growth medium. Primary cell cultures from human, guinea-pig, rabbit and mouse tissues were derived from single cell suspensions. These were prepared by mechanical chopping, followed by treatment for 15 minutes at 37° with 0·05 per cent (w/v) trypsin in 10^{-4} M EDTA, filtration through sterile gauze and plating in appropriate medium. To obtain large populations, the cell lines were grown in 8 oz. glass bottles at 37° in an atmosphere of 5 per cent CO_2 and 95 per cent air. For cell and colony counts, 60 × 15 mm. plastic plates (Falcon Plastics, Inc.) were used.

Antisera and Complement (C′)

Rabbit antisera to horse serum (anti-HS) and foetal calf serum were prepared by incorporation of the individual sera at 40 mg. protein per ml. into Freund's complete adjuvant and injection into the toe pads. Booster injections were given intravenously 4–6 weeks later, the animals bled the following week and the sera stored at −20°. Guinea-pig blood was obtained by heart puncture from 400 g. animals and the complement processed and stored according to the method of Kabat and Mayer (1961).

Double Diffusion in Gel

Both the Ouchterlony (1949) plate and the Preer (1956) tube methods were utilized to analyse the antibody–antigen precipitation reaction in agar.

Complement Fixation

For qualitative analysis, C′ block titrations with five $C'H_{50}$ per reaction mixture and three $C'H_{50}$ per control were analysed as described in Kabat and Mayer (1961). Quantitative C′ fixation was performed by the method of Mayer, Osler, Bier and Heidelberger (1948). The quantitative curve used for the detection of horse serum antigens was determined with fifty $C'H_{50}$ and 1·0 ml. of 1:50 R34 anti-horse serum antiserum. The lower

limits of detection of antigen, based on 7 per cent protein in horse serum, was 0·1 μg. of horse serum protein. Since the standard curve obtained was the summation of many antibody–antigen systems, it was used only for estimates and not for absolute values.

RESULTS

RETENTION OF SERUM COMPONENTS BY HeLa CELLS

The initial observation on the retention by cells in culture of serum protein components of the growth medium were made with a cloned HeLa line grown in parallel in horse serum and heated rabbit serum. The cloned line was obtained from the original HeLa cell population by the successive isolation of two colonies, each with about fifty cells. The cells were dispersed in medium containing either 20 per cent horse serum (these cells will be designated HeLa-H) or 20 per cent heated rabbit serum (HeLa-R).

HeLa-H and HeLa-R differed in both cell morphology and colony formation. The HeLa-R cells were large, coarsely granular, pigmented and formed loosely packed colonies as distinct from the relatively small, finely granular cells and syncytial-like colonies of HeLa-H. The colonial and the cellular morphological differences were readily interconverted by switching media. These observations are similar to those made by several other workers (Puck, Marcus and Cieciura, 1956; Rose, 1962) on the variation of cell morphology with serum.

Prior to immunization of rabbits with HeLa-H and HeLa-R an analysis was made of the ease and extent of removal of serum proteins from the cells by washing and treatment with trypsin. The cells were grown in 8 oz. bottles to a level of about 10^6 cells per bottle. The medium in each bottle was carefully removed, 10 ml. of saline G added and allowed to remain for several minutes with intermittent shaking, then removed. After three such washings, 10 ml. of 0·05 per cent (w/v) trypsin was added and removed, the cells incubated for 5 minutes at room temperature, collected in 10 ml. of saline G and immediately centrifuged and again washed by centrifugation with 10 ml. of saline G. At this point microscopic examination and cell counts revealed the onset of extensive disintegration of the cells and the procedure was terminated. The cells and wash fluids were analysed for horse serum with rabbit anti-horse serum antiserum (anti-HS) by means of gel diffusion and quantitative complement (C′) fixation. The results of this analysis are presented in Table 1. Included in the table are the amounts of residual horse serum expected by dilution calculated on the basis of 7 per cent protein in horse serum and an entrained volume of 0·5 ml. for each 10 ml. of wash fluid.

Examination of the data reveals that after six washings there still remained on the HeLa-H cells about 5·5 μg. of horse serum protein per 10^6 cells. The protein was easily demonstrable by quantitative C′ fixation, with 10^6 cells fixing about forty of fifty $C'H_{50}$ used in the analysis.

In the wash fluids no horse serum was detected by gel diffusion in the fourth and subsequent washes. With C′ fixation, however, the fifth and subsequent wash fluids were found to contain a constant level of horse serum protein, about 0·3 μg./ml., as if the protein were being eluted from the cells or contributed by disintegrating cells. It must again be stressed that the levels of horse serum protein found by C′ fixation are not absolute values but represent an order of magnitude. This derives from the fact that the rabbit anti-HS contained a number of antibodies of different specificity and it cannot be inferred that the

TABLE 1

ANALYSIS OF HeLa CELLS AND WASH FLUIDS FOR HORSE SERUM PROTEIN (HS) BY QUANTITATIVE C′ FIXATION AND GEL DIFFUSION

	No. of bands by gel diffusion	*Residual HS found by C′ fixation*	*Residual HS calculated (μg./ml.)*
HeLa-H			
Wash No. 1	5–7	—	—
Wash No. 2	3	—	35
Wash No. 3	1–2	—	1·8
Wash No. 4	0	—	0·1
Wash No. 5	0	0·34 μg./ml.	0·005
Wash No. 6	0	0·20 μg./ml.	0·0002
Wash No. 7	0	0·30 μg./ml.	0·00001
HeLa-H cells (after six washes)	—	5·5 μg./10^6 cells	—
HeLa-R cells (after six washes)	—	0 (<0·05 μg./10^7 cells)	
HeLa-R washes No. 1 to No. 7	0	0 (<0·05 μg.)	

Tabular values are averages of three independent experiments.

residual horse serum protein components measured on the cells or in the wash fluid are present in the same proportions as in the horse serum used as antigen to obtain the standard curve.

That the reaction between washed HeLa-H and anti-HS was caused by horse serum bound to the cells and not by cross-reaction between HeLa cells and horse serum was strongly supported by the evidence obtained with HeLa-R cells. With 10^7 HeLa-R cells as antigen, ten-fold greater than the number of HeLa-H cells used in the reaction, no C′ fixation was observed with anti-HS.

ANTIGENICITY OF HORSE SERUM BOUND TO HeLa CELLS

HeLa-R and HeLa-H cells were harvested, washed six times as described in the previous section and each of the populations injected intravenously into six rabbits. The primary schedule consisted of nine injections for HeLa-H and twelve injections for HeLa-R, three per week, with 10^6 cells per injection. The route of immunization and the relatively light schedule were chosen to minimize the possible contributions of the horse serum components. Five weeks after the termination of the primary course, two injections were given on successive days with a total of 2×10^6 cells. The animals were bled on the 7th day following the last injection.

Despite extensive washing of the HeLa-H cells and the light immunization schedule, antibody was formed to horse serum components. Analysis of the anti-HeLa-H antisera by gel diffusion revealed the presence of one band to horse serum in each of the six antisera, and there was an additional band formed by two antisera. The bands in the Ouchterlony plates were faint and diffuse and took from 48 to 72 hours to become visible. In Preer tubes the bands were visible in 24 hours and, while faint, were less diffuse than those in the plates. Absorption of the anti-HeLa-H antisera with horse serum prior to diffusion eliminated band formation. None of the pre-immune sera formed bands with horse serum nor did any of the antisera prepared against HeLa-R cells.

The presence of antibodies to horse serum components in anti-HeLa-H antisera was confirmed by complement fixation. In a block titration with a single antiserum, R61, and with a pool (R60-65) of the six anti-HeLa-H antisera a standard fixation pattern was obtained at antiserum dilutions of 1 : 2 and 1 : 5, with maximum fixation occurring at a horse serum dilution of 1 : 5000. No fixation was observed with anti-HeLa-R antisera or with pre-immune rabbit sera. Quantitative data obtained as part of another experiment showed that with 1·0 ml. of a 1 : 10 dilution of pool R60-65 and 1·0 ml. of a 1 : 1000 dilution of horse serum (moderate antigen excess), twenty-two of fifty $C'H_{50}$ were fixed (Table 2).

An attempt was made to identify the anti-horse serum components in the antisera to HeLa-H cells by the use of immunoelectrophoresis with horse serum as the antigen. Pooled anti-HeLa-H and anti-HeLa-R antisera were compared with a potent antiserum to horse serum. No reaction was observed with anti-HeLa-R but anti-HeLa-H antiserum gave a band of identity with anti-horse γ globulin. The immunoelectrophoresis pattern is illustrated in Fig. 1. As yet there is no evidence on the nature of the second horse serum component that appears to bind to HeLa cells.

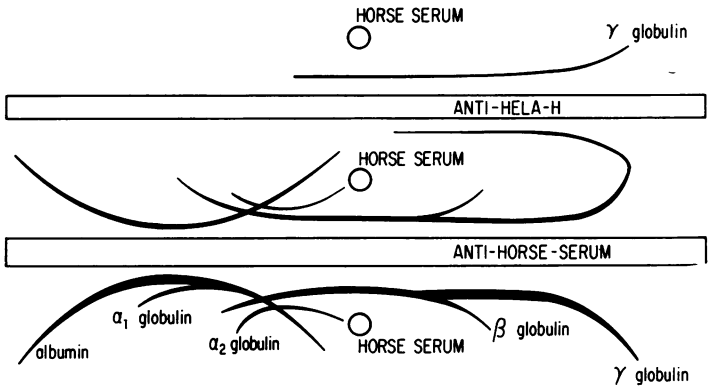

FIG. 1. Immunoelectrophoretic patterns of anti-HeLa-H and anti-HS reacted with horse serum. Labelled line drawing illustrating that the single band of identity is to the γ-globulin component of the horse serum.

The presence of anti-horse serum antibodies in anti-HeLa-H antiserum, and the binding of horse serum components by cells grown in horse serum, suggested that an unrelated cell line grown in horse serum could react with anti-HeLa-H by virtue of the common horse serum components. To test this possibility a primary cell strain was grown from foetal mouse tissue in horse serum medium. The cells were harvested, washed five times and examined for C′ fixation with anti-HeLa-H antiserum with and without the addition of a blocking level of horse serum. Any contribution of horse serum components on the mouse cells to C′ fixation would, in this way, be specifically eliminated by the presence of the blocking antigens. The data obtained in the experiment are presented in Table 2. The reaction between mouse cells (10^6) and anti-HeLa-H (1·0 ml.; 1:10 dilution) fixed forty of fifty $C'H_{50}$ after 20 hours at 2°. In the presence of horse serum (1·0 ml.; 1:5 dilution) fixation was reduced to six of fifty $C'H_{50}$, thereby implicating horse serum antigens on the mouse cells as the major contributor to the reaction. It may be pertinent that the amount of horse serum in the growth medium (20 per cent) is a satisfactory quantity for creating

antigen excess sufficient to prevent C′ fixation. Reaction tube 3 contained 1·0 ml. of the last wash fluid of the mouse cells and gave negligible fixation in support of the inference that the horse serum antigens were on the cells.

TABLE 2

REACTION OF MOUSE CELLS GROWN IN HORSE SERUM WITH ANTI-HeLa-H ANTISERUM

	1	2	3	4	5	6	7	8
Foetal mouse cells (10^6/ml.)	1·0 ml.	1·0 ml.	—	—	—	—	—	—
Foetal mouse cell supernatant	—	—	1·0 ml.	1·0 ml.	—	—	—	—
Horse serum (1 : 1000)	—	—	—	—	1·0 ml.	1·0 ml.	—	—
Blocking antigen (Horse serum 1 : 5)	—	1·0 ml.	—	1·0 ml.	—	1·0 ml.	—	1·0 ml.
$C'H^{33}$ fixed (of fifty $C'H_{50}$)	40	6	<4	<4	22	<4	0	0

Quantitative C′ fixation with anti-HeLa-H antiserum. Each reaction mixture contained 1·0 ml. of anti-HeLa-H (R60-65; 1 to 10), fifty $C'H_{50}$ and were made up to 10 ml. with veronal buffer. Fixation: 24 hours at 2°.

Preliminary experiments have been carried out with HeLa cells and a cloned primary human skin strain (TC 111961) grown in medium with foetal calf serum as the sole serum source. The data obtained suggest that, analogous to the binding of horse serum, at least one component of foetal calf serum is firmly bound to these cell strains.

IMMUNE INTERACTION BETWEEN HeLa-H CELLS AND ANTI-HORSE SERUM ANTISERUM

In the previous sections data were presented to show that serum components of the medium can bind firmly to animal cells and give rise to specific antibodies when the cells are injected into rabbits. The methods used to detect these antibodies were immunodiffusion and complement fixation. An additional and sensitive technique, commonly employed for detection of antibodies to cellular antigens, involves 'immune kill' of the cells by antiserum and complement. An analysis was, therefore, performed on the interaction between HeLa-H, anti-horse serum antiserum and guinea-pig complement.

HeLa-H and HeLa-R were grown in 8 oz. bottles, washed three times with 10 ml. saline G, collected with trypsin, centrifuged, washed and suspended in saline G. The cell count was determined in a haemocytometer. Reaction mixtures containing 5×10^3 cells, four $C'H_{50}$ and 1·0 ml. of the appropriate dilution of antiserum or normal serum were set up in a total volume of 2·2 ml., in duplicate, in 40 ml. sterile siliconized centrifuge tubes and incubated at 37° for 90 minutes with intermittent shaking. The reaction mixture was then brought up to 10 ml. with 23 per cent foetal calf serum in M199 and aliquot volumes plated, in triplicate, in 60 × 15 mm. plastic plates. After incubation for 4–7 days, the plates were fixed in absolute methanol, stained with Geimsa and counts made of total cells and colonies. Since total cell and total colony counts were found to be proportional, either or both were determined in a given experiment.

A measure of the error involved in the analysis is revealed by the data in Table 3 obtained from four separate reaction mixtures with HeLa-R, rabbit serum and C′. The error in sampling from one tube ranged from 5 to 15 per cent, while the error between replicate treatments, in this case about 4 per cent, generally did not exceed 15 per cent. A difference of 20 per cent was arbitrarily chosen as the significance level.

In Table 4 are recorded the results of an experiment with anti-HS and pre-immune serum on HeLa-H and HeLa-R. Included are the results simultaneously obtained with

antiserum to HeLa cells. At dilutions of 1 : 20 and 1 : 30, 1·0 ml. of anti-horse serum antiserum had no effect on HeLa-R, but gave about 50 per cent kill with HeLa-H, as measured against pre-immune serum. In contrast to the effect of anti-horse serum on HeLa-H, 1·0 ml. of 1 : 150 dilution of antiserum to HeLa cells was sufficient for 100 per cent kill. The error involved in counting, diluting and plating two different cell populations accounts for the difference in absolute numbers of the surviving HeLa-H and HeLa-R populations presented in Table 4. However, this is not pertinent to the analysis, since 'kill' is measured within a population, not between populations.

TABLE 3

ESTIMATION OF THE ERROR IN 'IMMUNE KILL' EXPERIMENTS

Reaction mixture	*Plate A*	*Plate B*	*Plate C*	*Average*
1. R34 (1 : 150)	748	927	937	871 ± 115
2. R34 (1 : 60)	965	816	1004	930 ± 100
3. R34 (1 : 30)	829	863	920	871 ± 47
4. R62 (1 : 30)	875	914	929	906 ± 28
				894 ± 29

Total cell counts were obtained in each of three plates from four replicate treatments of HeLa-R cells (5×10^3), 1·0 ml. of varying dilutions of rabbit sera, and four $C'H_{50}$.

TABLE 4

THE REACTION OF HeLa-H AND HeLa-R CELLS (5×10^3) WITH R34 ANTI-HS ANTISERUM OR PRE-IMMUNE SERUM (1·0 ml.) AND C′ (FOUR $C'H_{50}$)

Serum	*HeLa-H*		*HeLa-R*	
	Cell count	*Per cent kill*	*Cell count*	*Per cent kill*
Anti-HS (R34) 1 : 20	120	60	169	0
Anti-HS (R34) 1 : 30	122	50	162	0
Pre-immune (R34) 1 : 20	295	—	174	—
Pre-immune (R34) 1 : 30	232	—	142	—
C′ Control	289	—	142	—
Anti-HeLa-R (R54-59) 1 : 150	0	100	0	100
Anti-HeLa-H (R60-65) 1 : 150	0	100	0	100

After 1 hour at 37°, the mixtures were diluted, samples plated, incubated for 4 days, stained and total cell counts recorded as the average from three replicate plates. Per cent kill is calculated relative to pre-immune serum.

The C′ dependence of at least a portion of the toxicity of anti-HS and also of the standard, normal rabbit serum, is illustrated in Table 5. In this experiment HeLa-H cells were treated with anti-HS (R40) and the corresponding pre-immune serum with and without four $C'H_{50}$, and the results obtained as average colony counts formed from the surviving cells. Without C′ the sera were indistinguishable in their effect on HeLa-H. With C′, 40 per cent of the cells survived to give colonies in the normal serum and 20 per cent in anti-HS antiserum.

Several repetitions of the experiment on 'immune kill' confirmed that anti-HS was without effect on HeLa-R. The results with HeLa-H, however, were variable. While kill was demonstrable in the majority of experiments (the range was from 93 to 27 per

cent) occasionally there was no demonstrable difference between the antiserum and pre-immune serum. These results led to an examination of the standard against which kill was measured, the pre-immune serum.

TABLE 5

ILLUSTRATION OF C′ DEPENDENT KILL IN BOTH IMMUNE AND PRE-IMMUNE SERA

	Anti-HS (*R40*)	*Pre-immune* (*R40*)
With C′	25	55
Without C′	131	136

10^4 HeLa-H cells incubated at 37° for 90 minutes with 1:10 dilutions of rabbit serum (R40) and eight $C'H_{50}$ (where indicated). Fixed and stained after 5 days' growth, colonies counted and recorded as the average of three replicate plates.

An analysis of five different normal rabbit sera, all obtained, processed and stored in an identical manner, illustrated the wide variability of normal rabbit serum in its reaction with HeLa-H (Table 6). With normal serum R52, as with foetal calf serum, the addition of C′ had no effect on the reaction of the serum with HeLa-H, whereas with R33, R34, R39 and R63, C′ dependent kill ranged from 28 to 50 per cent. Moreover, if foetal calf serum were used as standard, two of the five sera showed no C′ dependent kill, while the

TABLE 6

THE EFFECT ON HeLa-H CELLS OF TREATMENT WITH FIVE NORMAL RABBIT SERA IN BOTH THE PRESENCE AND ABSENCE OF COMPLEMENT

Pre-immune rabbit serum	*Average colony counts*		*C′ dependent kill* (%)	
	With C′	*Without C′*	*Relative to same serum without C′*	*Relative to FCS+C′*
R33	37	71	47	0 (<20)
R34	25	40	40	41
R39	31	43	28	26
R52	58	61	0	0
R63	29	58	50	32
FCS	42	44	0	—

Cells (5×10^3), sera (1·0 ml. of 1 : 10), C′ (four $C'H_{50}$) mixed for 1 hour at 37°; the mixture diluted, plated, survivors grown for 4 days, stained and counted. The average colony counts are derived from three replicate plates. Per cent kill is tabulated for each serum without C′ and for each serum relative to foetal calf serum (FCS).

other three ranged from 26 to 41 per cent. From these results it appeared that normal pre-immune serum was a somewhat uncertain standard against which to measure immune kill with relatively weak immune systems such as HeLa-H and anti-horse serum antiserum. While valid for the usual serological reactions, it is possible that normal serum can exert a multiplicity of effects on HeLa cells, particularly at the relatively high concentrations required. For this reason the argument can be made that the anti-horse serum antibodies are superfluous and that kill by anti-HS R40 and R34 as measured against pre-immune sera merely represents the differential toxicity of different lots of rabbit serum. A less

equivocal standard would be a given antiserum which had been absorbed with antigen, in this case, horse serum.

With the HS anti-HS C′ fixation curve as a guide, anti-HS was absorbed with horse serum at various points and examined for kill against HeLa-H. Preliminary results, obtained with absorption at a point in the broad equivalence zone, indicate that anti-HS kill can be reduced. The ratio of antigen to antiserum used was 1·0 ml. of a 1 : 6000 dilution of horse serum and 1·0 ml. of a 1:30 dilution of R34 anti-HS. The results of this experiment, given in Table 7, show that with the addition of the antigen, an average of 1406

TABLE 7

REDUCTION OF HeLa-H CELL KILL BY ABSORPTION OF ANTI-HORSE SERUM ANTISERUM WITH HORSE SERUM

Anti-HS 1 : 30 (*ml.*)	*HS* 1 : 6000 (*ml.*)	*Average total cells*
0·2	0·2	1877
0·5	0·5	1474
1·0	1·0	1406
1·0	0·0	670

10^4 HeLa-H cells were reacted for 90 minutes with four $C'H_{50}$ and each of the four reaction mixtures. After 4 days of growtb three replicate plates were stained for each, total cells counted and their averages recorded.

cells was obtained as against 670 cells without the addition of the absorbing antigen. In a comparable experiment performed with HeLa-R and anti-HS, absorption of anti-HS by horse serum had no effect. With this antiserum, then, absorption by horse serum removed C′ dependent toxicity only for HeLa-H, not HeLa-R, suggesting that antibodies to horse serum do play a role in immune kill of HeLa-H.

Absorption at other points on the curve, 'antibody excess', 'antigen excess' with either horse serum or specific horse serum fractions have yielded somewhat curious results which are still under investigation together with other aspects of kill by heterogeneous immune systems.

DISCUSSION

The study of cellular antigens and the establishment of antigenic relationships in organisms such as the bacteria and protozoa are facilitated by the ease with which large, cloned populations of these organisms can be obtained. The development of methods for isolating and cloning animal cells in tissue culture from heterogeneous inocula offers the promise of successfully duplicating these results with the individual cell types of higher organisms. However, certain precautions in the application of current tissue-culture methods must be observed. For example, clonal analysis and plating efficiency studies of primary cell platings from normal tissues in the rat have shown the existence of selective forces which can lead to the emergence of cells of a minority class (Zaroff, Sato and Mills, 1961). The properties of this class of cells may have little to do with the characteristic properties of the tissue of origin and may offer misleading information if used to compare different tissues (Sato, Zaroff and Mills, 1960). A different type of bias is evident from the data presented

in the present studies on the incorporation by tissue-culture cells of serum antigens from the growth medium. These incorporated serum antigens while not products of the cell genome can still act as cellular antigens. The evidence for this assertion with a cloned HeLa line grown in horse serum (HeLa-H) is: (1) The persistence of horse serum antigens (one identified as horse γ globulin) on the cells after extensive washing—to the point of disintegration of the cells; and (2) the production of antibody to at least one horse serum component in each of six rabbits immunized with the extensively washed HeLa-H cells and with a moderate immunization schedule. That these anti-HS specificities do not represent cross-reactions between HeLa cells and horse serum is affirmed by the observation that in none of the pre-immune sera, nor in any of the six antisera to the same HeLa line grown in rabbit serum, were these specificities found by either gel diffusion or C′ fixation.

The detection of horse serum antibodies in anti-HeLa-H antisera, both by gel diffusion and C′ fixation, required careful titration with the antigen. The concentration of horse serum in the medium, for example, represents antigen excess and gave no C′ fixation with anti-HeLa-H when the medium was used to examine anti-HeLa-H for antibodies to horse serum. We now have evidence that mouse and human primary cell strains can also bind horse and foetal calf serum and upon immunization give rise to antibodies to the serum.

It is of interest that a discussion of these data with Dr. William T. Murakami led him to examine some of his rabbit antisera to polyoma virus purified from cells grown in horse serum. By means of gel diffusion he was able to detect moderately strong bands to horse serum in his polyoma antisera.

The ability of animal cells to pick up serum antigens and to serve as a carrier for the production of antibody to these antigens could lead to confusion in establishing antigenic relationships among animal cell populations. In particular, it can be inferred that less extensive washing procedures and adjuvant-coupled immunization with large numbers of cells could produce antibody concentrations against serum antigens sufficient to react with other cell lines grown in the same serum. This may be one mechanism for the origin of common antigenic determinants observed after long-term cultivation *in vitro* (Coriell, Tall and Gaskill, 1958). Evidence in support of this suggestion can be seen in the results presented in Table 2. In this experiment it was shown that foetal mouse cells grown in horse serum and thoroughly washed react by C′ fixation with anti-HeLa-H antiserum and that this reaction is blocked by the addition of horse serum. In an anaslysi of cellular antigenic relationships by C′ fixation the growth medium would not serve as a control since the dilution of horse serum used as blocking antigen was equivalent to the amount commonly used in the growth medium.

The circumvention of this 'acquired' antigenic relationship by absorption is not a simple matter since more than one serum component is apparently involved. Unless each antibody is absorbed at equivalence, competition among competing equilibria in antigen excess may still give a reaction with test cells. A satisfactory way to eliminate the problem of acquired antigenic specificity might be to eliminate macromolecular constituents from the medium or to grow the cells in the serum of the animal to be immunized. Unfortunately, we were unable to devise a serum-free medium for the growth of any of our established lines or primary strains. Nor were we, in spite of a variety of treatments, successful in devising a rabbit serum medium for the long-term cultivation of non-rabbit primary strains, although the established lines, such as HeLa, can be grown in pooled heated rabbit serum.

These observations, together with those of Coombs *et al.* (1961) on the acquisition of

Forssman specificity by cells in tissue culture, emphasize the care that must be taken to define the potential antigenic contributions of the growth medium.

Another method commonly employed to demonstrate antigenic relationship is 'immune kill' by antiserum and C′. The question of whether or not rabbit anti-horse antiserum and C′ can kill HeLa-H cells has not been completely resolved. Some of the experimental difficulties involve the relatively high concentration of anti-horse antiserum required and, related, the standard against which kill is measured. With pre-immunization serum as standard, C′ dependent kill of from 30 to 80 per cent was obtained in the majority of experiments with antiserum dilutions of 1:30 to 1:10. In an individual experiment kill was proportional to antiserum concentrations. However, some fluctuation was encountered on experimental repetition and occasionally the difference in kill between immune and pre-immune serum was within experimental error. The high levels of serum required in this reaction (dilutions of 1:10 to 1:30 for immune anti-horse antiserum as compared with 1 : 150 for homologous anti-HeLa antiserum) led to an examination of the standard: normal rabbit serum. It became apparent that normal sera from different animals varied widely in C′ dependent kill of HeLa-H. We now have preliminary evidence that this variation is exhibited by separate bleedings from an individual animal and that an individual serum alters in this property through ordinary handling such as storage and freezing and thawing. Presumably this behaviour reflects the sensitivity of the cells to a multiplicity of reagents present in normal serum including natural antibody. The most reliable indicator of specific antibody in 'kill' experiments may then be the effect of a particular antiserum before and after absorption with antigen. On this basis, the results of the experiment on absorption of anti-HeLa-H by horse serum, recorded in Table 7, do suggest kill of HeLa-H cells by anti-horse antiserum. However, further analysis is required and studies are continuing in an attempt to clarify this reaction and to examine its significance and role in immune pathology *in vivo*.

ACKNOWLEDGMENTS

The authors wish to thank Dr. William O. Weigle and Nora Calaprice for the immunoelectrophoretic analysis, and to acknowledge the technical assistance of Amna Cornett, Leanne Hinton and Eleanor Gelatte. This research programme was supported, in part, by Grant No. RG-8269 from the National Science Foundation.

REFERENCES

COOMBS, R. R. A., DANIEL, M. R., GURNER, B. W. and KELUS, R. (1961). 'Recognition of the species of origin of cells in culture by mixed agglutination.' *Int. Arch. Allergy*, **19**, 210.

CORIELL, L. L., TALL, M. G. and GASKILL, H. (1958). 'Common antigens in tissue culture cell lines.' *Science*, **128**, 198.

GAILLARD, P. J. (1953). *International Review of Cytology*, vol. II, pp. 331–401. Academic Press, New York.

KABAT, E. A. and MAYER, M. M. (1961). *Experimental Immunochemistry*, 2nd ed., p. 224. Thomas, Springfield.

MAYER, M. M., OSLER, A. G., BIER, O. G. and HEIDELBERGER, M. (1948). 'Quantitative studies of complement fixation. I. A method.' *J. Immunol.*, **59**, 195.

MORGAN, J. F., MORTON, H. J. and PARKER, R. C. (1950). 'Nutrition of animal cells in tissue culture. I. Initial studies on a synthetic medium.' *Proc. Soc. exp. Biol. (N.Y.)*, **73**, 1.

OUCHTERLONY, O. (1949). 'Antigen–antibody reactions in gels.' *Acta path. microbiol. scand.*, **26**, 507.

PREER, J. R., JUNR. (1956). 'A quantitative study of a technique of double diffusion in agar.' *J. Immunol.*, **77**, 52.

PUCK, T. T., CIECIURA, S. J. and ROBINSON, A. (1958). 'Genetics of somatic mammalian cells. III. Long term cultivation of euploid cells from animal and human subjects.' *J. exp. Med.*, **108**, 945.

PUCK, T. T., MARCUS, P. I. and CIECIURA, S. J. (1956). 'Clonal growth of mammalian cells *in vitro*: Growth characteristics of colonies from single HeLa cells with and without a "feeder" layer.' *J. exp. Med.*, **103**, 273.

ROSE, G. G. (1962). 'The morphological diversity of Gey's strain HeLa after ten years in tissue culture.' *Texas Rep. Biol. Med.*, **20**, 308.

SATO, G., ZAROFF, L. and MILLS, S. E. (1960). 'Tissue culture populations and their relation to the tissue of origin.' *Proc. nat. Acad. Sci. (Wash.)*, **46**, 963.

ZAROFF, L., SATO, G. and MILLS, S. E. (1961). 'Single cell platings from freshly isolated mammalian tissue.' *Exp. Cell Res.*, **23**, 565.