observations, therefore, rule out the hypophyseal-cortical axis as a common mechanism.

Of even more interest is the effect seen when salicylate was given just prior to PCI. Here the inhibition of the edematous response usually produced by PCI was markedly reduced, if not lost all together. The interference by salicylate upon the PCI mechanism, as mentioned above, is one possibility for explaining this effect. An alternate possibility of this salicylate effect is that by reducing the inflammatory response to the first irritation the stimulus to the counter-irritation mechanism is sufficiently reduced as not to alter the second inflammation under the conditions of this study.

The existing evidence suggests that PCI leads to production of a generalized hyporeactivity to irritation. Local hyporeactivity at the site of irritation has been established (6,7,8,9). By altering or blocking the PCI mechanism, salicylates may allow a more rapid return to the initial reactivity. However, until the nature of this "protection" against inflammation afforded by PCI is established, it is difficult to evaluate the salicylate effect in this study.

Summary. The effects of sodium salicylate on prophylactic counter-irritation, accomplished by irritating the opposite ankle 15 hours prior to the test ankle, were studied in rats. It was found that: (1) The capacities to inhibit edema by prophylactic counter-irritation and sodium salicylate were similar; (2) Sodium salicylate administered 1 hour prior to irritation of the test ankle did not increase the degree of inhibition produced by prophylactic counter-irritation; and (3) Sodium salicylate given 1 hour prior to the prophylactic counter-irritation caused the latter's anti-edema effect to be lost.

1. Hamdy, M. K., Kunkel, L. E., Rheins, M. S., Deatherage, F. E., *J. Animal Sci.,* 1957, v16, 496.

2. Laden, C., Blackwell, R. Quentin, Fosdick, L. S., *Am. J. Physiol.,* 1958, v195, 712.

3. Bowman, D. C., Jr., Doemling, D. B., Harris, S. C., *Fed. Proc.,* 1960, v19, 87.

4. LaBelle, A., Tornaben, J. A., *Science,* 1951. v114, 187.

5. Kelemen, E., *Permeability in Acute Experimental Inflammatory Oedema in the Light of the Action of Salicylates,* Hungarian Acad. Sci., 1960.

6. Miles, A. A., Miles, E. M., *J. Physiol.,* 1952, v118, 228.

7. Hoene, R., Labbe, P., Selye, H., PROC. SOC. EXP. BIOL. AND MED., 1952, v79, 65.

8. Arteta, J. L., *ibid.,* 1954, v85, 162.

9. Salgado, E., Hoene, R., *ibid.,* 1954, v86, 190.

Received July 31, 1961. P.S.E.B.M., 1961, v108.

Species-Related Antigens of Mammalian Cell Strains as Determined by Immunofluorescence.*† (26960)

CYRIL S. STULBERG, WILLIAM F. SIMPSON, AND LAWRENCE BERMAN
Child Research Center of Michigan, and Dept. of Pathology, Wayne State University College of Medicine, Detroit

In the development of characterizing procedures for cultured cell strains, identification of their species of origin is of primary importance, especially since it is commonly known that some cell strains have become contaminated with others of diverse parentage. This problem has been approached in several ways, including chromosomal analyses(1), virus susceptibility(2), and immunologic identification(3-4). In an effort to identify cell strains in a manner analogous to the systematic serology long employed in

* This study was supported by Grant from Nat. Cancer Inst.

† Presented in part at April 1961 meeting of Fed. Am. Soc. Exp. Biol.

TABLE I. Cell Strains Used for Production of Labeled Antisera.

Cell strain	*Species and tissue of origin*	*Characterizing properties*
Detroit 6*	Human, sternal marrow	Epithelial-like, heteroploid, virus susceptibility
HeLa 229†	Human, CA of cervix	idem
Detroit 504*	Human, teratoma of testes	Fibroblast-like, diploid, virus susceptibility
LLC-MK_2‡,**	Monkey, kidney	"Epithelial-like," virus susceptibility
S-180§	Mouse, sarcoma 180	"Fibroblast-like," tumorigenic
16F-FAF 28‖	Chinese hamster, peritoneal elements	"Fibroblast-like," karyology

* Cell strains originated in these laboratories.
† Provided by Dr. J. T. Syverton, Univ. of Minnesota, Minneapolis.
‡ " " Dr. R. N. Hull, Eli Lilly Research Laboratories, Indianapolis, Ind.
§ " " Dr. G. E. Foley, Children's Cancer Research Foundation, Boston, Mass.
‖ " " Dr. G. Yerganian, " " " " " " "
** Previously LLC-MK_2 (NCTC 3196, Evans), cultivated herein in serum medium.

microbiology, this report describes the development of an immunofluorescence method for rapid and specific identification of cultured mammalian cells.

Materials and methods. Cell strains. A variety of mammalian cell strains originally derived from several different animal and tissue sources(5-7) were selected for preparation of antisera. These strains, representing 4 animal species are listed in Table I with information pertinent to their sources and characteristics. These and several additional types of cell cultures were used for the fluorescent antibody tests, namely, Detroit-508, a fibroblast-like strain derived from human kidney; CCRF 228 and 232‡(8), strains derived from mouse lungs (the former from a C57 BL/6 and the latter from a DBA/1 mouse); and primary cultures of Rhesus monkey kidney cells.

Preparation and assay of antisera. The cell strains listed in Table I were used to immunize guinea pigs according to the procedure of Brand and Syverton(3). It was felt that antisera prepared in this manner could be used to verify species origin of cells by their ability specifically to agglutinate erythrocytes of the homologous species(3). It was presumed that such hemagglutinating antibodies could be used as a guide for the efficiency of subsequent conjugating procedures. The various cell strains were cultured as monolayers in 6-oz. prescription bottles, employing Eagle's minimal essential medium(9) and supplemented with 10% human serum for human-derived strains and 5% calf serum for the others. Twenty-four hours prior to harvesting, all monolayers grown in medium with human serum were washed, then re-fed with medium containing 5% calf serum.

To harvest cells, all monolayers were rinsed once with serum-free medium, the cells removed from the glass by scraping, and the dispersed cells washed in 2 additional changes of serum-free medium. Total cell yields from 6 replicate cultures, amounting to 6 to 8 $\times$ 10^7 cells, were suspended in 6.5 ml of serum-free medium. Of this, 3.5 ml was emulsified with equal parts of Freund's complete adjuvant. Each of 6 guinea pigs were injected subcutaneously at 4 sites on the abdomen, using 0.25 ml of the emulsion at each site. Simultaneously, each animal received 0.5 ml of cell suspension (without adjuvant) intraperitoneally. The combination of injections was equivalent to approximately 1 $\times$ 10^7 cells per animal. Animals were bled by cardiac puncture before, and 4 weeks after injections. In some instances, attempts were made to enhance the antibody titers (specific hemagglutinins) by weekly intramuscular injections of cell suspensions but no appreciable differences could be observed either in titers or specificity of the antisera. The sera (inactivated at 56°C. for 30 min-

‡ Provided by Dr. G. E. Foley, Children's Cancer Research Foundation, Boston, Mass.

TABLE II. Hemagglutination Characteristics of Various Cell Antisera.

Species Erythrocytes	Hemagglutinin titer * Whole serum						Hemagglutinin titer *† after conjugation with Fluorescein Isothiocyanate					
	Detroit -6	HeLa	Detroit -504	LLC-MK_2	Mouse S-180	Hamster	Detroit -6	HeLa	Detroit -504	LLC-MK_2	Mouse S-180	Hamster
Human	512	256	1024	64	64	32	32	32	16	0	4	0
Monkey (Rhesus)	32	128	32	512	8	0	0	8	0	16	0	0
Mouse	0	0	4	4	16	32	0	0	0	0	0	0
Hamster ‡ (Chinese)						64						0
Rabbit	4	0	0	0	0	4	0	0	0	0	0	0
Guinea Pig	0	0	0	0	0	0	0	0	0	0	0	0
Sheep	0	0	0	0	0	0	0	0	0	0	0	0

* Reciprocal of dilution.
† Not corrected for dilution effects.
‡ Hamster RBC spontaneously agglutinated with most guinea pig sera.

utes) from animals inoculated with a given cell strain were assayed, both individually and in pools, by determination of hemagglutinins for erythrocytes corresponding to the species of origin of the cell strains, according to the procedure of Brand and Syverton(3). The hemagglutinin titers of the various antiserum pools are shown in Table II. The species specificity of the antisera to the cell strains of human origin (Detroit-6, HeLa, Detroit-504), and the cell strain of monkey origin (LLC-MK_2), as well as the common antigenic component between the 2 species (3) are evident. On the other hand, antisera to the mouse and Chinese hamster strains yielded inconclusive results with regard to hemagglutinins.

Preparation of fluorescent antibodies. The antisera to be conjugated were fractionated either with half saturated ammonium sulfate or 26% sodium sulfate and dialyzed by standard procedures. Globulins thus obtained were conjugated with fluorescein isothiocyanate (purified, obtained from Baltimore Biological Laboratories), by adding the powdered dye to the globulin solutions at pH 9 with stirring in an ice bath and followed by dialysis against phosphate buffer, pH 7.2(10). The conjugates were stored frozen until used.

Hemagglutinin titers of the conjugates are illustrated in Table II. The conjugation procedure resulted in a general loss in hemagglutinin titer, but the specificities of the conjugates as compared to the whole sera appeared to be retained. In addition, whenever, the serum hemagglutinin titers were low, the corresponding conjugates exhibited little or no hemagglutinin.

Staining procedure. The cells of monolayer cultures were dispersed with 0.25% trypsin solution and the resulting cell suspensions were washed 3 times with buffered saline, pH 7. An alternative procedure involved preservation of cell suspensions(11) by slow freezing in glycerol-serum media, storage at −190°C., rapid thawing, and 4 subsequent washings. The latter method proved to be valuable by eliminating the necessity for preparation of large numbers of cultures of various strains and their possible cross-contamination just prior to staining, while yielding identical results. Each cell suspension was brought to approximately 2% concentration by volume in buffered saline, pH 7.4. One-tenth ml of cell suspension was mixed with 0.1 ml of labeled antibody in the wells of non-wettable plastic slide plates which were then placed in a humidity chamber on a rotary shaker and agitated for 30 minutes at room temperature. Stained suspensions were then transferred to small tubes, and washed with 3 changes of buffered saline. Following the last wash, the supernate was decanted, and the cells resuspended in the fluid that remained. One drop of this suspension was placed on a slide (not exceeding 1 mm in thickness), covered with a #1 coverslip, gently pressed with blotting paper, and sealed with clear finger-nail lacquer. Such preparations were examined within 2 hours by fluorescence microscopy,

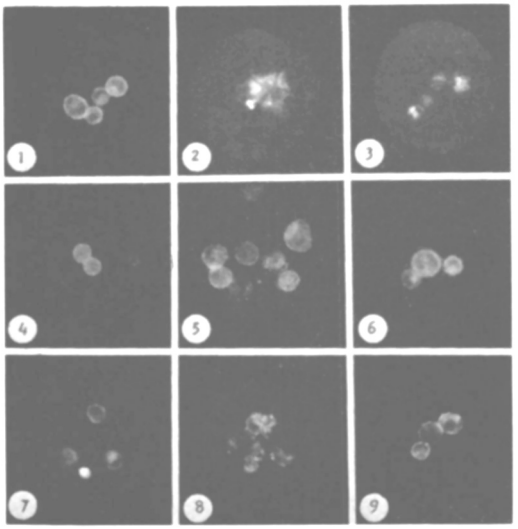

FIG. 1. Detroit-6 cells stained with anti Detroit-6 conjugate.
FIG. 2. LLC-MK_2 cells stained with anti Detroit-6 conjugate.
FIG. 3. Chinese hamster cells stained with anti Detroit-6 conjugate.
FIG. 4. HeLa cells stained with anti HeLa conjugate.
FIG. 5. Detroit-504 cells stained with anti HeLa conjugate.
FIG. 6. Detroit-6 cells stained with anti HeLa conjugate.
FIG. 7. S-180 cells stained with anti S-180 conjugate.
FIG. 8. LLC-MK_2 cells stained with anti S-180 conjugate.
FIG. 9. Chinese hamster cells stained with anti Chinese hamster conjugate. (All photomicrographs are at 300 × total magnification)

using a BG-12 pass filter and an OG-1 barrier filter. This staining procedure was suggested by the work of Cohen, *et al.*(13) who demonstrated blood group antigens by peripheral staining of erythrocytes with fluorescein-labeled antibodies.

Observations and results. The interpretations of the staining reactions were based entirely on presence or absence of *peripheral* fluorescence of the cells. Fig. 1 illustrates a group of cells stained with homologous labeled antibody, and provides an example of what will be referred to as "specific" or "positive" staining. Additional examples of specific staining of the various cell strains are shown in Fig. 4-7 and 9. The intensities of the positive reactions varied to some extent from conjugate to conjugate. Brilliant staining was encountered when labeled Detroit-6, HeLa, or Detroit-504 antibodies were applied to Detroit-6 and HeLa cells (Figs. 1, 4, 6). However, each of these labeled antibody preparations produced a thin but well-defined periphery of fluorescence with Detroit-504 cells (Fig. 5), although the latter was difficult to reproduce photographically. At the other extreme, 2 of the conjugates, namely anti S-180 and anti Chinese hamster, produced weaker but nevertheless specific peripheral staining with their homologous cell preparations (Fig. 7, 9). The conjugates also exhibited some variation with regard to the number of cells that appeared unstained in otherwise positive homologous antibody-cell systems; an estimated 1-5% of the cells did not exhibit peripheral fluorescence in a given preparation. The incidence of the latter appeared to be inversely related to intensity of the staining. Care was taken to distinguish true peripheral staining of individual cells from that which occurred when conjugate was trapped between cells in instances where large clusters or clumps were present.

Negative reactions were defined as those in which no peripheral fluorescence was evident (Fig. 2, 3, 8). In these instances the cells exhibited mottled or solid fluorescent areas that could be distinguished from positive reactions.

Cross-reactions between antibody preparations for cells of human and monkey origin observed in the hemagglutination reactions (Table II) were also evident by staining with fluorescent antibodies. It was important therefore, to attempt selective absorption of the cross-reacting antibodies involved. Absorption with cultured cells was not feasible at the time because of the unavailability of the quantities of cells needed. However, since erythrocytes had been shown to be effective in removing cross-reacting hemagglutinins between these two species(3), it was felt that erythrocytes might also be effective for absorption of those conjugates which exhibited hemagglutinin activity. Thus, the conjugates made against the Detroit-6 and LLC-MK_2 strains were absorbed § with Rhesus monkey or human O negative red cells. The results

§ The conjugates were absorbed twice with one volume of packed red cells to 2 volumes of conjugate for 30 minutes in the cold.

TABLE III. Effect of Absorption on Cross-Staining Between Fluorescent Antibodies and Cells of Human and Monkey Origin.

	Staining titer * of labeled globulins					
	Unabsorbed		Absorbed with human RBC		Absorbed with monkey RBC	
Cell strain	Detroit-6	LLC-MK_2	Detroit-6	LLC-MK_2	Detroit-6	LLC-MK_2
Detroit-6 (Human)	8	2	2	0	2	0
LLC-MK_2 (Monkey)	4	4	0	2	0	2
Sarcoma 180 (Mouse)	0	0				
CCRF 228 "	0					
CCRF 232 "	0					
Chinese Hamster	0	0				

* Reciprocal of dilution.

are shown in Table III. Although these absorptions eliminated the cross-staining between the fluorescent antibodies of the 2 species, paradoxically, absorption with either species of red cells rendered the staining reactions specific. The explanation for the latter phenomenon is not clear.

In Table IV are shown the staining characteristics of the various absorbed conjugates for cells of homologous and heterologous species origin. The labeled antibodies prepared against the Detroit-6, HeLa, or Detroit-504 strains reacted specifically only with cells of human origin. Similarly, the labeled antibody against the monkey kidney strain was positive not only for that strain, but for primary cultures of monkey kidney cells as well. The S-180 labeled antibodies produced recognizable peripheral staining of S-180 cells, but when tested against the 2 mouse strains of normal tissue origin, the results were somewhat evanescent, possibly because this antibody preparation was weak (Fig. 7). The fluorescent antibodies prepared against the Chinese hamster strain were also specific only for those cells. With the exception of Detroit-508, each combination was repeated at least several times, and where sufficient labeled antibody was available (Detroit-6, HeLa, LLC-MK_2), these were used as additional controls with homologous and heterologous cells with each combination tested. There were no instances where discrepancies occurred with the combinations shown in Table IV.

Discussion. Current problems in cell research demonstrate the need for methods that will distinguish cell strains with respect to their species-related antigens. For the purposes of a cell culture collection, it was necessary to develop a practical method for rapid and direct identification of cell strains to be characterized. Of the various immunological studies that have been reported, the work of Brand and Syverton, involving indirect hemagglutination(3), and that of Coombs et al., involving the mixed agglutination technique(4), have offered the most promising approach to this problem. Both of these methods involve detection of species antigens

TABLE IV. Staining Characteristics of Various Fluorescein-Labeled Antisera.

	Staining reactions with labeled antibodies					
Cells	Detroit -6	HeLa	Detroit -504	LLC-MK_2 (Monkey)	S-180 (Mouse)	Chinese Hamster
Detroit-6 (Human)	+	+	+	0	0	0
HeLa 229 "	+	+	+	0	0	0
Detroit-504 "	+	+	+	0	0	0
Detroit-508 "	+	+	+	0	0	0
LLC-MK_2 (Monkey)	0	0	0	+	0	0
Primary kidney "	0	0	0	+	0	0
S-180 (Mouse)	0	0	0	0	+	0
CCRF 228 "	0	0	0	0	±	0
" 232 "	0	0	0	0	±	0
Chinese hamster	0	0	0	0	0	+

common to both red cells and cultured tissue cells. The experiments described herein suggest that fluorescein-labeled antibodies may provide a means for directly identifying species-related antigens on cell surfaces.

To test the feasibility of the procedure, antibodies were prepared against cell strains whose origins were reasonably certain on the basis of one or more identifiable "marker" characteristics, *i.e.*, morphology, karyology, tumorigenicity, or virus susceptibility spectrum. Moreover, the origins of several of the strains were verified by the hemagglutination method, and all were verified by the results of the fluorescent antibody tests. Nevertheless for preparation of reference antisera, the use of primary cultures might be preferable, principally to obviate questions of source or antigenic changes. The latter is currently under investigation. Although the immunization procedures employed herein involved the use of guinea pigs, the latter were not wholly satisfactory, primarily because of the weak antibody responses to cells of mouse or hamster origin, as measured both by hemagglutinins and by fluorescent antibodies.

The data suggest that hemagglutination and fluorescence may be detecting different groups of antibodies, since specific fluorescence was obtained in the absence of hemagglutination. This made it difficult to assess the efficiency of the conjugation procedures, although it is quite clear that 26% sodium sulfate was unsatisfactory for serum fractionation since marked loss of titer occurred due to dilution effects.

It is likely the fluorescent antibodies in the stained preparations were reacting with antigens at the cell surface, analogous to the agglutination reactions studied by Kite and Merchant(12), and the mixed agglutination reaction of Coombs et al.(4). It should be emphasized that the staining reactions took place on surfaces of live cells in suspension. The use of dried or fixed smears or monolayers was entirely unsatisfactory because dead or non-viable cells stained non-specifically. The cells in the preparations were spherical and it was presumed that the exposed antigenic sites were concentrated over a minimal surface area of the contracted cell in contrast to cells attached to glass where the cell surfaces would be spread out over a much greater area.

Summary. A procedure has been described whereby species-associated antigens were directly identified in cultured mammalian cells by fluorescent antibody techniques. Guinea pig anti-cellular globulin fractions were conjugated with fluorescein isothiocyanate. Dispersed cells were prepared from monolayer cultures or frozen cell suspensions and the resulting wet cell suspensions were treated with the labeled antibodies. Staining specificity was recognized by presence or absence of peripheral fluorescence of the treated cells. Four cell strains derived from human tissues, 3 from mouse, and one from Chinese hamster, reacted specifically with homologous but not with heterologous labeled antibodies. Antibodies to monkey- and human-derived strains exhibited reciprocal cross-reactions that were eliminated by absorption procedures.

1. Hsu, T. C., *Internat. Rev. Cytol.*, 1961, in press.

2. Hull, R. N., Tritch, O. J., *J. Nat. Cancer Inst.*, 1961, in press.

3. Brand, K. G., Syverton, J. T., *ibid.*, 1960, v24, 1007.

4. Coombs, R. R. A., Daniel, M. R., Gurner, B. W., Kelus, A., *Immunol.*, 1961, v4, 55.

5. Berman, L., Stulberg, C. S., Ruddle, F. H., *Blood*, 1955, v10, 896.

6. Hull, R. N., Cherry, W. R., Johnson, I. S., *Anat. Rec.*, 1956, v124, 490.

7. Foley, G. E., Drolet, B. P., PROC. SOC. EXP. BIOL. AND MED., 1956, v92, 347.

8. ———, ———, McCarthy, R. E., Goulet, K. A., Dokos, J. M., Filler, D. A., *Cancer Res.*, 1960, v20, 930.

9. Eagle, H., *Science*, 1959, v130, 432.

10. Cherry, W. B., Goldman, M., Carski, T. R., *P.H.S. publication #729*. U. S. Govt. Printing Office, Washington, D. C., 1960.

11. Stulberg, C. S., Peterson, W. D., Berman, L., *J. Nat. Cancer Inst.*, 1961, in press.

12. Kite, J. H., Merchant, D. J., *ibid.*, 1961, v26, 419.

13. Cohen, F., Zuelzer, W. W., Evans, M. M., *Blood*, 1960, v15, 884.

Received July 31, 1961. P.S.E.B.M., 1961, v108.