

TISSUE CULTURE STUDIES ON HUMAN SKIN

II. COMPARATIVE EFFECTS OF CERTAIN SPECIFIC CONTACT ALLERGENS ON SENSITIZED AND NON-SENSITIZED HUMAN SKIN*

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The skin occupies an important position in the study of allergy. Sulzberger has aptly stated that, "The degree of general significance and the exact extent of the importance of the skin as an organ exercising peculiar immunobiologic functions is not yet known. On the other hand, there can be no doubt whatsoever that the skin as a register and a recording organ has come to occupy a unique position in the study of allergy" (1).

The purpose of the present study is to determine whether or not there is any difference in the toxicity of representative contact allergens for human epithelial cells growing in tissue culture when comparing individuals who are not sensitive to the contactant under investigation with individuals who are sensitive. The toxicity of the allergen for the skin explant is determined by its capacity to cause inhibition of outgrowth of the epithelial cells. In planning this study, it was thought that these experiments might indicate whether the specific reactivity of the skin involved in allergic contact type eczematous dermatitis is connected with the skin itself or dependent on other factors.

Contact type hypersensitivity of the skin has been investigated extensively in the past several decades. Jadassohn (2) in 1895 was the first to develop the concept of testing the capacity of the skin to react by means of local application of suspected allergens, which is the basis for the patch test as it is used in practice. Since that time, numerous workers have expanded and developed this fundamental principle. Bloch's (3) work was especially noteworthy in this field. In this country, Sulzberger and his co-workers (4-6) have done pioneer work on contact type eczematous dermatitis; one of the numerous important contributions of this group was the compilation of data regarding the appropriate concentrations of drugs and chemicals to be used in conducting patch tests in the study of individual cases. It is not within the scope of this paper to review the extensive data which have been accumulated in this field.

With the advent of the tissue culture method by Harrison (7) (1907), Carrel and Burrows (8) (1910), it became possible to study living tissues growing in-vitro. During the intervening years, methods have been developed and technics perfected whereby pure cultures of practically every cell type found in the animal body have been grown and maintained in an artificial medium for varying periods of time (9, 10). Human skin is included among the tissues successfully grown outside the body. It produces migrating sheets of typical epithelioid cells which can be observed and studied in the living state.

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In 1922, Ebling and Fisher (11) described the growth of chick epithelium in tissue culture. Bornstein (12) in 1930, and Pinkus (13) in 1932, observed cultures of normal human skin over periods of several weeks; they noted migration and growth of true epithelial cells. Parshley and Simms (14, 15) have studied the nutritional requirements of skin explants in tissue culture. They reported that one of their nutrient solutions suppressed the growth of fibroblasts and favored the growth of epithelial cells, and another favored the growth of fibroblasts and inhibited the epithelial cells. Other pertinent reports include the papers of Lewis, Pomerat, and Ezell (16), who studied living cultures of human epidermal cells with phase contrast microscopy, and Ulloa-Gregori, Blocker, Nowinski, and Pomerat (17), who compared the growth promoting properties of various body fluids for epithelial cells growing in tissue culture.

In a previous study by the authors (18), it was demonstrated that the outgrowth of human epithelial tissue in tissue culture is uniform and in general quite constant. However, for reasons which were not apparent, considerable variations in growth potential were found to exist in explants obtained from different individuals although all cells of explants from the same individual tended to migrate at approximately the same rate and in a similar manner. Furthermore, although outgrowth can be maintained for only 8 to 9 days, it was possible to make quantitative measurements and record the degree of inhibition which resulted from the addition of various chemicals and drugs to the culture medium. It was also established that this inhibition of outgrowth was dependent on the particular chemical or drug under investigation and that a given concentration regularly produced the inhibition regardless of the source of the explant. In that study (18), the toxicity for epithelial cells of eight commonly used local medicaments was determined, expressing the results in micrograms/ml of culture medium.

Additional reports pertinent to this study include those of Rich and Lewis (19, 20), Moen (21), Aronson (22), Raffel (23) and Landsteiner and Chase (24, 25). These workers studied the effect of tuberculin on explants of spleen, bone marrow, and leukocytes from tuberculous and non-tuberculous guinea pigs and rabbits. All of these investigators reported that the migration of cells in tissue culture explants of spleen, bone marrow, and leukocytes was inhibited by a given concentration of tuberculin which did not interfere with the growth of tissue culture explants of the same tissues taken from non-tuberculous animals. Blatt and Nantz (26), in a study of blood leukocytes in the evaluation of bacterial hypersensitivity of the tuberculin type, reported that the inclusion of a given concentration of *H. Streptococci* filtrate in the tissue culture medium resulted in the degeneration of leukocytes obtained from patients sensitive to *H. Streptococci*, while leukocytes from patients who were not sensitive to streptococci were not affected.

MATERIALS AND METHODS

Human skin used in this study was obtained by the following method. The skin of the forearm was thoroughly scrubbed with soap and water. Alcohol (70%) was poured over the area to be used and allowed to air dry. The skin was then compressed between the rubber covered blades of a sterile intestinal clamp (see Fig. I). A thin section of skin was then removed from the clamped area by means of a sterilized razor blade. This tissue was transferred to a Petri dish containing a small amount of Gey's balanced salt solution (Cameron (9)). Using the method established by Lewis, Pomerat, and Ezell (16), the skin so obtained was set up in the following manner (see Fig. II):

1. Fragments approximately 2 mm. square were cut for explanting.
2. These explants were imbedded in a clot composed of 1 drop of cockerel plasma containing 1000 units of penicillin/ml (final concentration of 250 units/ml of culture medium) and 1 drop of embryonic extract containing the allergen to be tested.

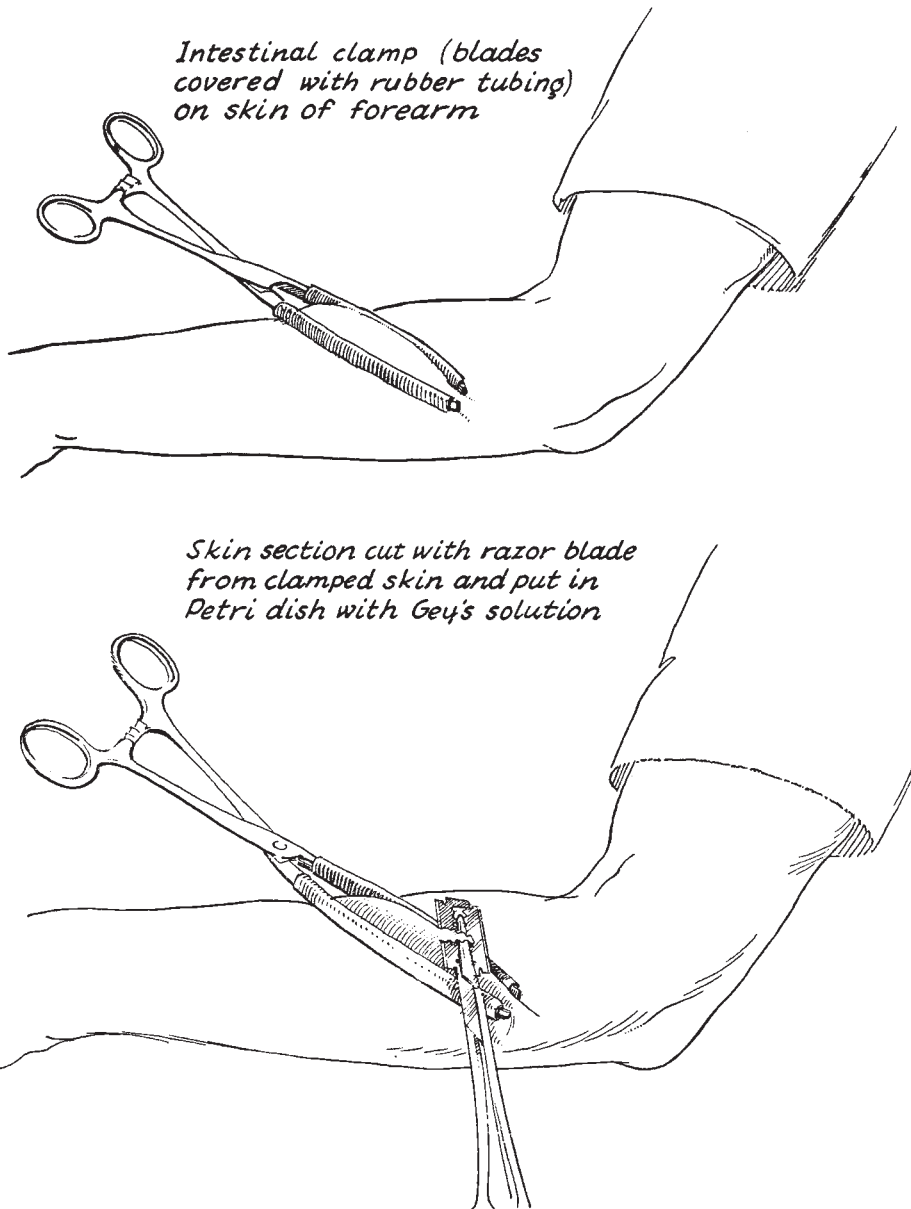
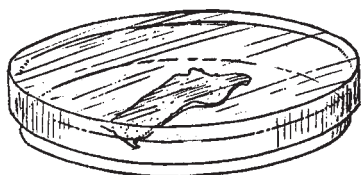


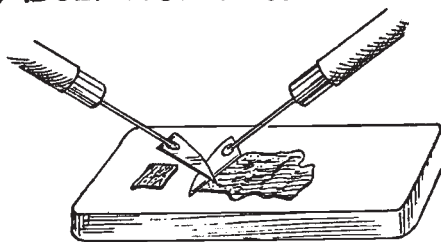
FIG. 1. Skin obtained by clamping area to be used (forearm) with intestinal clamp, blades of which are covered with rubber tubing. Thin section of skin removed from clamped area with a razor blade.

3. After the clot had set firmly, two drops of malignant ascitic fluid were added.
4. These clots were made on cover glasses, and after the ascitic fluid was added, they were mounted on a hanging drop slide, sealed with paraffin, and incubated at 37°C. for 8 days. Each day the cultures were examined microscopically and the amount of outgrowth recorded.

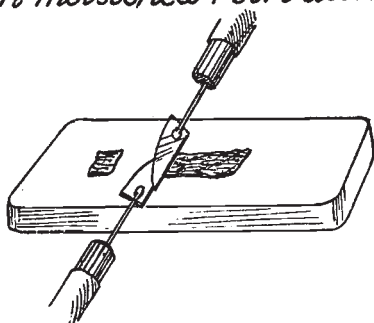
STEPS IN CULTURING ADULT HUMAN EPIDERMIS



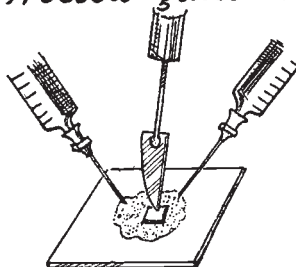
A. Skin from surgery in moistened Petri dish



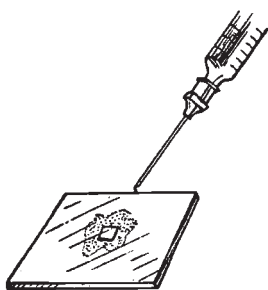
B. Trimming edges of skin on a "frosted" glass slide.



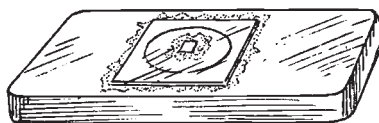
C. Skin cut in 2mm squares.



D. Skin imbedded (b) in a clot formed of chick embryo extract (a) and cockerel plasma delivered from 27 gauge needles attached to tuberculin syringes



E. After clot has "set," 2 drops of cell-free malignant ascitic fluid is added to the surface.



F. The cover glass is sealed with paraffin and incubated at 37° C as a "lying drop."

FIG. II

Migration of epithelial cells from human skin usually began in 48 to 72 hours after the experiment was set up and continued until the 5th or 6th day. Out-growth was graded by estimating the amount of the low power field it covered.

Studies of control tissues indicated that skin taken from patients in fair to good nutritional balance and which was cut so that the epidermis and only a very thin layer of dermis was included would produce an outgrowth of migrating

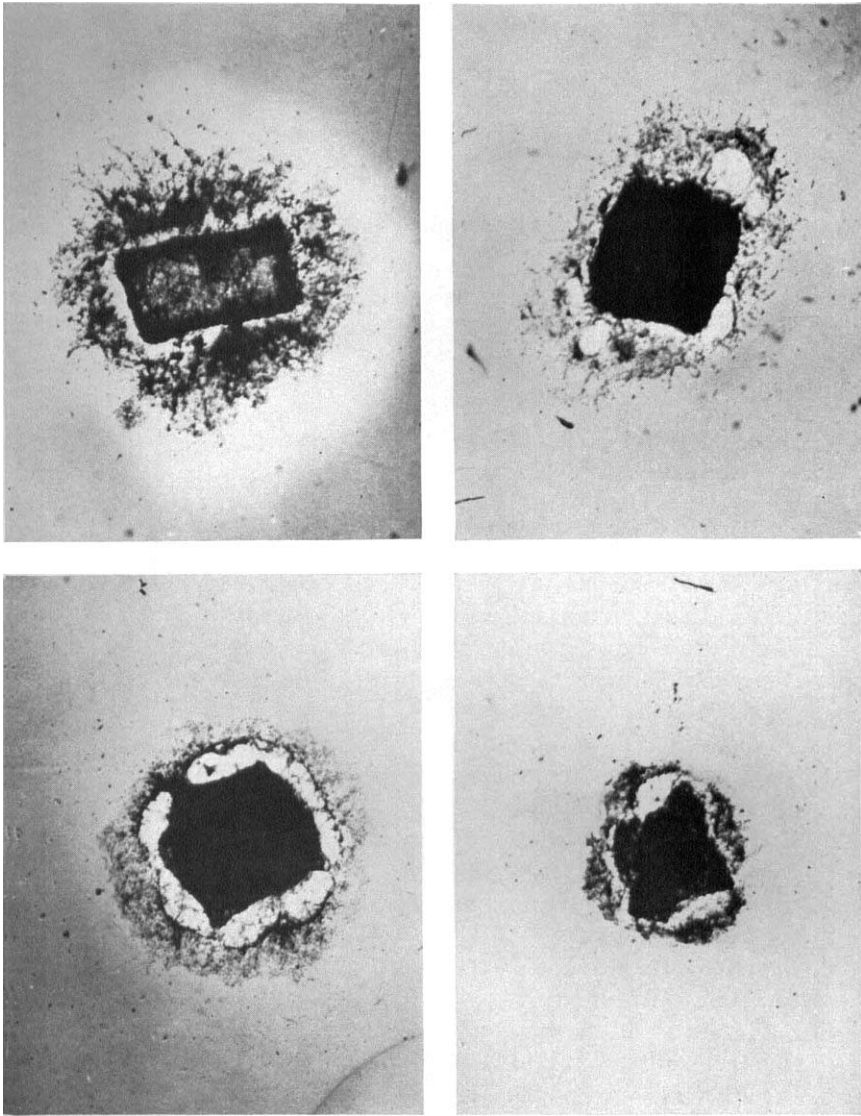


FIG. III. Explants of human skin grown in tissue culture. Upper left hand figure graded 4-plus, upper right 3-plus, lower left 2-plus and lower right 1-plus.

epithelial cells which was equal in width to the diameter of the low power field ($10\times$ objective, $10\times$ ocular— $100\times$) in from 5 to 7 days. Such an outgrowth was considered an average growth and rated 4-plus. A cellular sheet extending

over only one-fourth of the low power field was graded 1-plus, and sheets which covered one-half and three-fourths of the field were graded two and three plus respectively (see Fig. III). Migration usually stopped after the 6th day, but cultures were read daily until the 8th day, at which time evidence of degeneration began to appear. Control explants were set up in each case, and those experiments in which the controls did not attain at least a 3-plus outgrowth were considered unsatisfactory and not taken into account in the summary of results.

Three contact allergens were chosen for this study:

1. Oleoresin of *Rhus toxicodendron*
2. 2:4-dinitrochlorobenzene
3. Old tuberculin.

The minimal inhibitory dose (MID) and least injurious dose (LID) were determined for each compound. This was done according to the method previously described (18), e.g., the skin explants were imbedded in clots containing varying concentrations of the substances until the smallest quantity required to effect total inhibition of outgrowth was determined. This quantity was designated as the minimal inhibitory dose or MID. Similar titrations indicated the smallest quantity required to produce the first evidence of injury as compared to an untreated control. This quantity was labeled as the least injurious dose or LID.

For *Rhus* oleoresin, 2:4-dinitrochlorobenzene, and old tuberculin, the MID and LID were determined for skin explants from patients known to be non-sensitive to these compounds on patch test. After determining these concentrations, skin explants from patients known to be sensitive on patch test to the three allergens were exposed to the MID and LID concentrations and an intermediate quantity between these two ranges. Cultures were observed for the amount of outgrowth in each case.

The number of patients in the different categories was as follows:

1. Twelve patients patch test negative to *Rhus* oleoresin with a total of 310 cultures.
2. Eight patients patch test positive to *Rhus* oleoresin with a total of 225 cultures.
3. Fifteen patients patch test negative to 2:4-dinitrochlorobenzene with a total of 390 cultures.
4. Five patients patch test positive to 2:4-dinitrochlorobenzene with a total of 140 cultures.
5. Six patients with negative patch tests and negative intradermal tests to tuberculin with a total of 225 cultures.
6. Five patients with positive patch tests and positive intradermal tests to tuberculin with a total of 175 cultures.

For the determination of sensitivity to *Rhus* oleoresin, standard patch tests were used with an ether extract of *Rhus toxicodendron** in acetone in concentrations of 1:100 and 1:1000. All of the eight sensitive patients reacted positively to the 1:100 solution, and two were positive to the 1:1000 solution.

* Supplied by Graham Laboratories, Dallas, Texas.

Sensitivity to 2:4 dinitrochlorobenzene was induced by the method described by Sulzberger and Rostenberg (6) in which a drop of a 1% solution was applied to the forearm. The patients were tested for sensitivity in seven to twenty-one days after application and, if negative, were re-tested after re-application of the solution.

"Tuberculin Old" (Eli Lilly and Co. V-810) was used for determining sensitivity and for incorporation in the tissue culture medium. Patients listed as negative to old tuberculin showed negative reactions to 1:10,000 Volmer patch tests and to 1:100 intradermal injection of old tuberculin. All tuberculin positive patients gave a 3-plus reaction to a 1:10,000 Volmer patch test of old tuberculin.

Stock solutions of the allergens were made in acetone and dilutions prepared from these stock solutions in Gey's Balanced Salt Solution (9). Toxicity studies on acetone alone revealed that it was non-toxic for skin in the concentrations which were used.*

TABLE I

Minimal inhibitory dose and least injurious dose of Rhus oleoresin, 2:4-dinitrochlorobenzene, and old tuberculin for tissue culture explants of human skin

COMPOUND	PATCH TEST REACTION	NUMBER OF PATIENTS	NUMBER OF EX-PLANTS	MID	LID
<i>Rhus oleoresin</i>	Negative	12	310	250-500 µg/ml	25-50 µg/ml
<i>Rhus oleoresin</i>	Positive	8	225	250-500 µg/ml	25-50 µg/ml
2:4-dinitrochlorobenzene	Negative	15	390	12.5-25 µg/ml	1.25-2.50 µg/ml
2:4-dinitrochlorobenzene	Positive	5	140	12.5-25 µg/ml	1.25-2.50 µg/ml
Old tuberculin	Negative	6	225	40-50 mg/ml	10-12.5 mg/ml
Old tuberculin	Positive	5	175	40-50 mg/ml	10-12.5 mg/ml

RESULTS

The minimal inhibitory dose (MID) of *Rhus oleoresin* for skin explants obtained from non-sensitive subjects was found to be between 250 and 500 micrograms/ml of culture medium and the least injurious dose (LID) between 25 and 50 micrograms/ml of culture medium. Skin from patients who exhibited positive patch test reactions to *Rhus oleoresin* gave identical results, showing complete inhibition of outgrowth between 250 and 500 micrograms and injury between 25 and 50 micrograms/ml of culture medium.

The MID for 2:4-dinitrochlorobenzene for skin explants from non-sensitive individuals was between 12.5 and 25 micrograms/ml and the LID between 1.25 and 2.5 micrograms/ml of culture medium. Skin explants from sensitive patients also showed complete inhibition between 12.5 and 25 micrograms and first visible injury between 1.25 and 2.5 micrograms/ml of culture medium.

The MID for old tuberculin in the case of tuberculin negative patients was

* MID for acetone between 1:5 and 1:10; LID between 1:60 and 1:80. Highest concentrations used in these experiments 1:140.

between 40 and 50 milligrams (1:20–1:25) per ml of culture medium and the LID between 10 and 12.5 milligrams/ml of culture medium (1:80–1:100). Tuberculin positive patients exhibited the same degree of inhibition, with the MID between 40 and 50 milligrams and the LID between 10 and 12.5 milligrams/ml of culture medium. The above data are summarized in Table I.

DISCUSSION

Raffel (23) has reviewed the basic differences between the delayed and immediate types of hypersensitivity. These differences are based on the following criteria:

1. Time—the immediate type of response occurs within minutes after contact with the allergen, while in the delayed type, hours intervene between contact with the antigen and the allergic response.
2. Type of response—the smooth muscle and vascular endothelium make up the shock organ in the immediate type of hypersensitivity, while no such shock organ exists for the delayed type.
3. Presence of antibodies—these have never been demonstrated in the delayed type of hypersensitivity, either by serological test or by passive transfer of hypersensitivity.

Landsteiner and Chase (24) in studies of sensitization of animals with simple chemical compounds, by intracutaneous injection of the allergen, were able to induce sensitivity to 2:4 dinitrochlorobenzene of both the immediate and the delayed type. From these experiments, they suggested the tentative explanation that cutaneous manifestations are due to antibodies, perhaps of a special sort, produced by and fixed in the skin. Furthermore, they speculated from experiments with conjugated antigens that sensitization may be due to the combination of the antigen with proteins of the body.

In a study to investigate whether specific reactivity is connected with the skin itself or with humoral factors, Haxthausen (27) in 1948 used a special skin grafting technic. He found that skin grafts from allergic eczematous recipients, while still in the growing phase, gave negative results when treated with specific allergens. However, some time later, after the grafts had healed completely, they reacted just as did the remainder of the skin of the host. In other words, if the recipient had a positive reaction to the allergen elsewhere on the body, the healed skin graft also reacted positively, and if the recipient had a negative reaction to the allergen at other sites on the skin, although it had come from a positive donor, the grafted skin became negative on the negative host. He drew the following conclusions from this experiment:

1. New, rapidly growing skin will not react to an allergen, regardless of the sensitivity of the donor or the recipient of the skin.
2. Grafted skin, after complete healing, acquires the characteristic reaction of the recipient, regardless of the presence or absence of sensitivity in the donor. He also postulated that reactivity of the skin to allergens is due to a humoral factor, rather than any intrinsic alteration in the epithelial cells themselves.

The present study regarding the effect of antigens on explants of human skin in tissue culture seems to be in agreement with Haxthausen's work, in that the growing skin from both sensitive and non-sensitive patients reacted similarly. In other words, the presence in the culture medium of a contact allergen to which the individual was sensitive did not result in the inhibition of the outgrowth of the epithelial cells to any greater degree than was the case for explants obtained from an individual who was not sensitive to the contactant.

SUMMARY

1. Explants of human skin in tissue culture were exposed to *Rhus* oleoresin, 2:4-dinitrochlorobenzene, and old tuberculin.

2. The minimal inhibitory dose (MID) and least injurious dose (LID) of *Rhus* oleoresin, 2:4-dinitrochlorobenzene, and old tuberculin were determined for skin explants obtained from sensitive individuals and compared with explants obtained from non-sensitive individuals.

3. No difference could be observed between the amount of outgrowth of skin from sensitive and non-sensitive patients when these allergens were included in the culture medium.

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DISCUSSION

DR. PECK, *New York*: The work on tissue transplants was quite remarkable, and it gives us one more weapon, of course, to try to answer the question of whether we can passively transfer epidermal sensitivity.

I think it is a beautiful piece of work and it should be carried further, but it seems interesting in the light of the paper of Baer and Sulzberger to see that they were able to demonstrate inhibition of growth with the leukocytes for tuberculin sensitivity and not with actual epidermal cells. And now, they show that the leukocytes are important in what seemed to me was an apparent active sensitization. But I think the second paper especially was a very important one from a very practical viewpoint. It is not seldom that I have a patient receive a transfusion and develop a transfusion reaction with the acquisition of a host of sensitivities which persist. It was very hard to explain. I think in the light of this work, I am going to suggest to sensitive patients, at any rate, that instead of getting whole blood transfusions where possible, they receive pooled plasma.

DR. H. PINKUS, *Detroit*: My comment will be on the technic of Dr. Everett's work rather than on the results of her study. Nobody who has not attempted tissue culture of human epidermis can realize from the simple way in which Dr. Everett presented her findings how much effort is behind this work. I am very much pleased to hear that Dr. Pomerat and his collaborators have now worked out a simple method with which epidermis will proliferate in tissue culture, and which gives results reliable and predictable enough so that the cultures can be used for experimental purposes, even if only for a few days. I believe that the success of these workers is in great part due to the fact that they wisely restricted their efforts to short-term culture while previous workers, including myself, tried in vain to arrive at permanent pure strains of cells as a supposed prerequisite of exact experimentation. I like to ask Dr. Everett two specific questions. How did you overcome liquefaction of the clot? And didn't you encounter too much cornification? The tendency to excessive cornification was in my experience the greatest obstacle to long continued culture of human epidermis (Ueber Gewebekulturen menschlicher Epidermis. Arch. für Dermat. und Syph. **165**: 53, 1932).

DR. BURGOON, *Philadelphia*: I should like to congratulate Dr. Livingood and his group for adapting this tool as an investigative procedure in dermatology. The cytologist has studied living cells in tissue culture for many years but dermatologic exploitation has been lacking.

In recent years, the virologist has used this medium to study the effect of viruses on the living cell. In the past two years, our group in the Skin Virus Laboratory at Children's Hospital of Philadelphia have been doing the growth curves and histochemical procedures to determine the effect of the Herpes Simplex Virus on the rabbit corneal epithelium.

I am particularly happy to see this interesting study on the effect of contact allergens on sensitized human epithelial cells. This work demonstrates that there is more to epithelial sensitization than is immediately obvious. Further elucidation of the effect of antigens on sensitized epithelial cells might be obtained through phase microscopy to observe the mitochondria and by pyronin-methyl-green studies to study the effect on the ribosenucleic acid and desoxynucleic acid.

DR. BEERMAN, *Philadelphia*: My remarks are in no way a criticism of any of the presentation or any of the discussors, but I would like to keep the record straight.

Tissue culture as a tool in dermatology was studied by Dr. Hermann Pinkus a number of years ago. I also did a little tissue culture work (but not with skin) in 1924 with Dr. Leo Loeb. Since Dr. Burgoon may have made some people misunderstand—tissue culture is not a new tool, but one which is now being used in our field after the pioneer work of Dr. Pinkus and Dr. Pomerat.

DR. LIVINGOOD, *Galveston*: I wish to thank the discussors for their comments. In answer to Dr. Beerman, the history in regard to the in-vitro culture of human

skin is included in our paper. Because of lack of time, it was not possible for Dr. Everett to read the entire paper as it will appear in print.

It would appear from our results that young epithelial cells growing in tissue culture do not have the capacity to react to specific contact allergens. As far as I know, no one has devised a method of growing epithelial cells for more than ten to twelve days; perhaps the cells would acquire the capacity to react in a specific manner if they could be maintained in culture for a longer period of time. In this connection, it is interesting to note that Haxthausen with his homologous grafting experiments in identical twins concluded that new growing epithelium did not react to specific contactants, but sometime later after the grafts had healed completely, these sites reacted in the same manner as the other parts of the skin of the recipient.

We are in the process of collecting data on the toxic effect of various drugs, antibiotics, and chemicals on epithelial cells growing in tissue culture. Our results to date indicate that this method of approach may be a useful one in regard to predicting the potential irritant effects of new and old topical medications. Thus far there is a striking correlation with the in-vivo effects as evidenced by clinical experience with the topical agents under investigation as well as patch test results. In addition, it is feasible to determine the possible protective effect of other drugs and biologic agents as well as the possible synergistic toxic effects of various combinations of drugs. This work will be reported at a later date.

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