DESTRUCTION OF EPIDERMAL CELLS IN VITRO BY AUTOLOGOUS SERUM FROM NORMAL ANIMALS*

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Although normal serum is sometimes said to be toxic to autologous cells *in vitro* (1), to our knowledge, data of such autotoxic effects have been previously reported only for spermatozoa (2, 3). Indeed, autologous serum is often used as the best nutrient medium for cells (4). Evidence will be presented here that fresh *normal* sera from rabbits and rats are lethal *in vitro* to *autologous* epidermal cells. In the test system employed, 70 to 100 per cent of epidermal cells were killed within 2 hours at 37°C.

Materials and Methods

Animals.—The following strains were used: Dutch and Zealand rabbits, Long Evans rats, C57 mice, and mongrel guinea pigs.

Cell Preparation.—Epidermal cells from the basal or malpighian layer of the epidermis were prepared by the trypsinization method of Billingham and Reynolds (5) with a few modifications. A 0.010 to 0.015 inch split thickness of skin was obtained from the lateral surface of the body with a Brown electrodermatome. It was then scraped free of dead cuticular cells and connective tissue, and placed with its dermal surface down in a Petri dish containing 0.25 per cent trypsin (Difco 1:250) in versene-BSS (0.002 M tetrasodium ethylenediaminetetraacetate in Hanks's balanced salt solution). Upon incubation in a shaker at 37°C for 20 minutes, areas of the skin which appeared clear were excised and the remaining skin allowed to incubate for periods up to 1½ hours. The trypsinized pieces were then washed in saline and in versene-BSS, placed with the dermis of the skin up in a dry Petri dish, and blotted free of fluid. After peeling back the dermis the exposed surface of the epidermis was scraped with a scalpel. The freed malpighian cells were suspended in versene-BSS, pooled, centrifuged at 1000 *g* for 1 minute, and resuspended in BSS. Later tests have indicated that the use of versene could be eliminated completely.

In Vitro Cytotoxicity Tests.—In a glass tray having 10 mm × 2 mm concavities, 50,000 to 100,000 epidermal cells were added together with 0.2 ml of serum dilutions in BSS (without $NaHCO_3$). Upon incubation in a moist chamber at 37°C for 2 hours, viability of cells was assessed by eosin dye exclusion (6). For each determination, 300 cells were counted under oil immersion with phase contrast illumination. Toxicity was expressed by the cytotoxic

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index (C.I.):

$$C.I. = \frac{C - E}{C}$$

in which C = per cent viable cells in control serum (serum heated at 56°C for 30 minutes), and E = per cent viable cells in experimental serum.

Depletion of Complement.—Fresh normal rabbit serum was depleted of complement components by varying quantities of antigen-antibody precipitate, zymosan, or NH_4OH. Rabbit antiserum to bovine serum albumin (BSA) was reacted with BSA at equivalence for 1 hour to form the precipitate. Upon washing twice in the cold, an aliquot of the precipitate was taken for nitrogen analysis with biuret (7). Zymosan, prepared in the standard manner (8), was incubated with serum for 1 hour with shaking at 10 minute intervals. Since hydrazine was toxic to epidermal cells, C'_4 was inactivated by addition of NH_4OH. Complement levels were determined by standard methods employing sheep red blood cells sensitized with rabbit hemolysin (9). Measurements of hemolysis with a Beckman/Spinco model 151 spectrophotometer were used to calculate the complement levels in 50 per cent hemolytic units. Zymosan and inulin were obtained from Nutritional Biochemicals Corporation, Cleveland; ε-amino-*n*-caproic acid (EACA) and bovine fibrinolysin from California Corporation for Biochemical Research, Los Angeles; and soybean trypsin inhibitor from Sigma Chemical Company, St. Louis.

RESULTS

Autotoxicity of Serum.—The sera of rabbits and rats were extremely toxic to the malpighian or basal cells of the animals' own epidermis. Of a total of 43 rabbits tested, a mean cytotoxic index (C. I.) of 0.87 was found at a 1:2 dilution of autologous serum. Sera from 11 rats were 67.4 per cent toxic to autologous epidermal cells. In contrast, mouse sera were inactive (mean C.I.=0.04; range −0.13 to 0.4 in 9 animals). That autotoxic effects are not produced by a *lack* of some essential substance *in vitro* is shown by the observation that dilution of rabbit or guinea pig serum resulted in diminished toxicity (Fig. 1).

Toxicity of autologous serum was maximal when approximately 1 million epidermal cells were incubated with 1 ml of serum. As the number of cells was increased to 46 million per ml of serum, toxicity steadily declined (Fig. 2). It is perhaps important to note that the absolute number of cells killed per ml of serum was not constant, but was proportional to the number of cells used in the test. All tests to be described were carried out at the effective ratio of about 1 million cells per ml.

Inactivation of Complement.—Autotoxicity of rabbit sera was abolished by heating at 52°C or 56°C for 10 minutes (Fig. 3). Absorption of rabbit complement with increasing amounts of BSA–anti-BSA precipitate led to a gradual fall in complement levels which was paralleled closely by a fall in autotoxicity to epidermal cells. Two experiments yielded similar results (Fig. 4). Absorption of C'_3 with increasing zymosan concentrations also produced a corresponding decrease in toxicity together with loss of C' activity (Fig. 5). Further, at 3°C, inulin in a concentration of 20 mg/ml absorbed toxicity of serum as well as C'

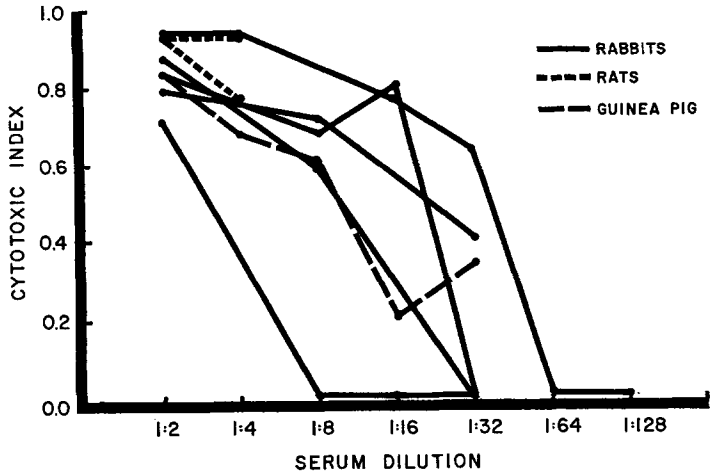

FIG. 1. Toxicity of fresh autologous serum dilutions upon epidermal cells.

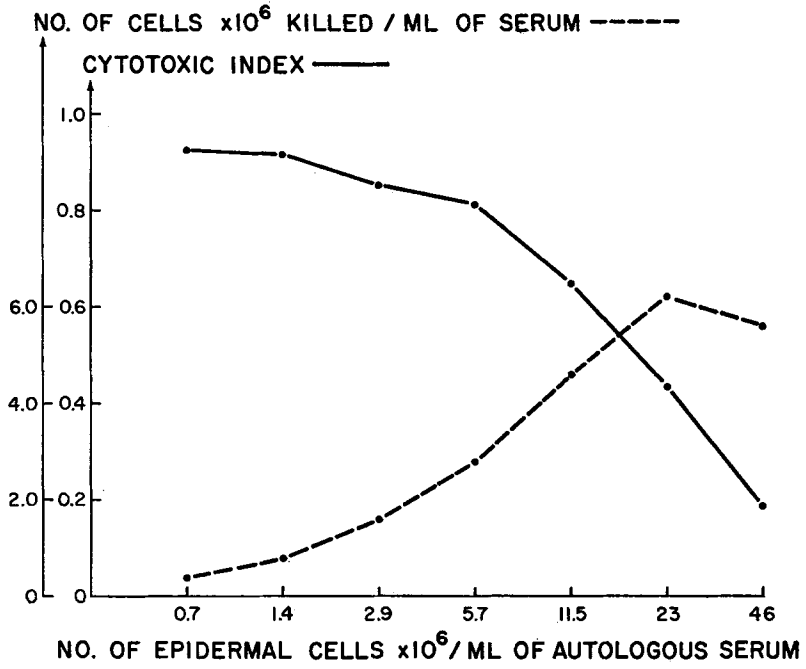

FIG. 2. The effect of cell:serum ratio upon autotoxicity.

activity. Serum similarly absorbed for 1 hour with 10 mg/ml of inulin retained its toxicity to malpighian cells, but also contained 19 units of complement per ml. Addition of NH_4OH to serum followed by incubation for 1½ hours reduced complement levels of serum as well as toxicity to epidermal cells (Fig. 6). In conjunction with this experiment, serum depleted of C'_3 (R3) was added in

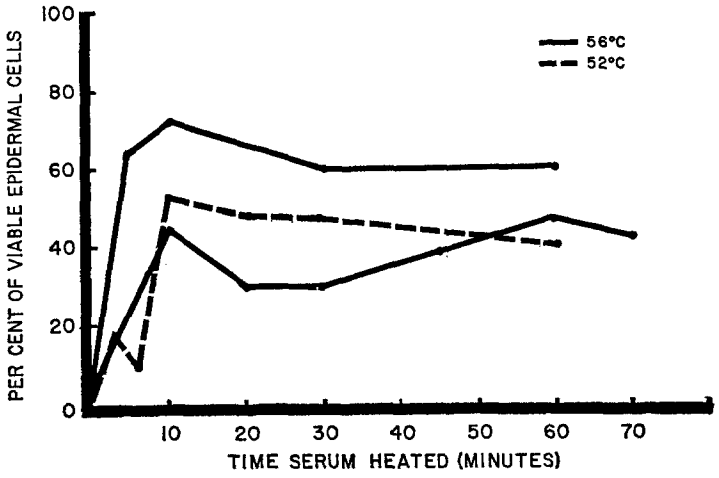

FIG. 3. Effect of heating serum upon autotoxicity.

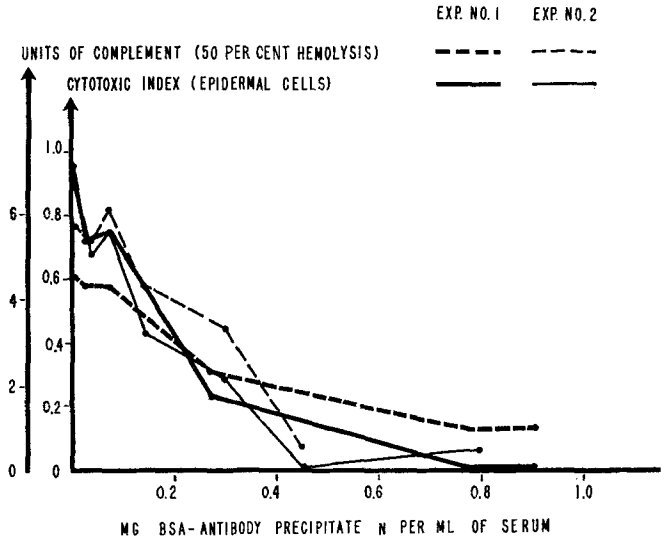

FIG. 4. Absorption of complement with BSA–anti-BSA precipitate and the loss in autotoxicity to epidermal cells.

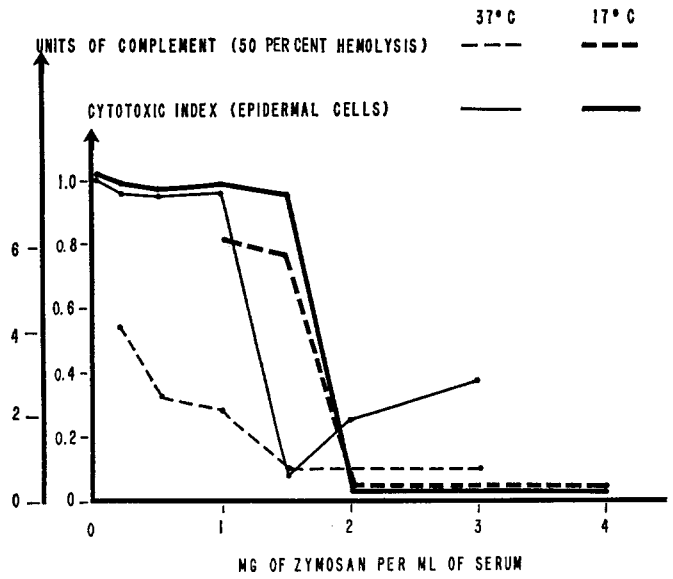

FIG. 5. Absorption of complement with zymosan and the loss of autotoxicity to epidermal cells.

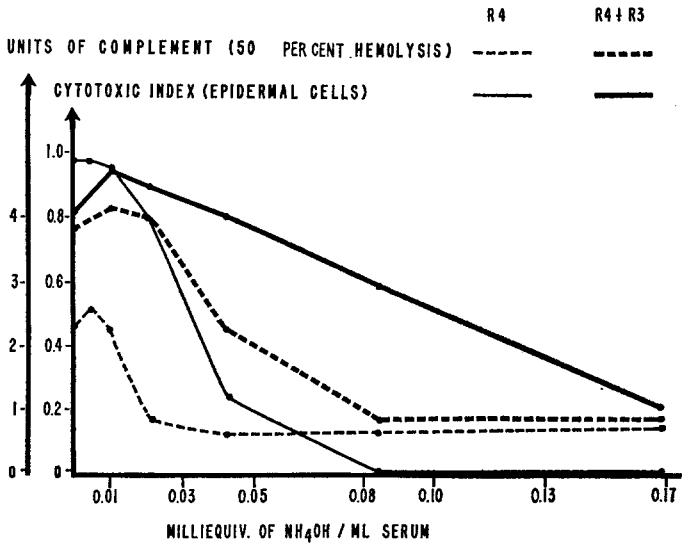

FIG. 6. Depletion of C'_4 with NH_4OH and the recombination of R4 and R3; their effects upon complement levels and upon autotoxicity to epidermal cells.

constant amounts to an aliquot of serum treated with NH_4OH (R4). As a result, together with the restoration of complement activity, cytotoxicity to autologous basal cells was regained (Fig. 6). Versene at levels which abolish C′ activity was injurious to epidermal cells. Attempts to split and recombine the end-piece and mid-piece of C′ were unsuccessful at the high concentrations of serum required to demonstrate toxicity.

From a preliminary test, it appears that malpighian cells "fixed" the greatest amount of *autologous* rabbit complement of the tissues tested. When expressed as units of C′ fixed per million cells, spleen cells fixed 0.025; lymph node cells,

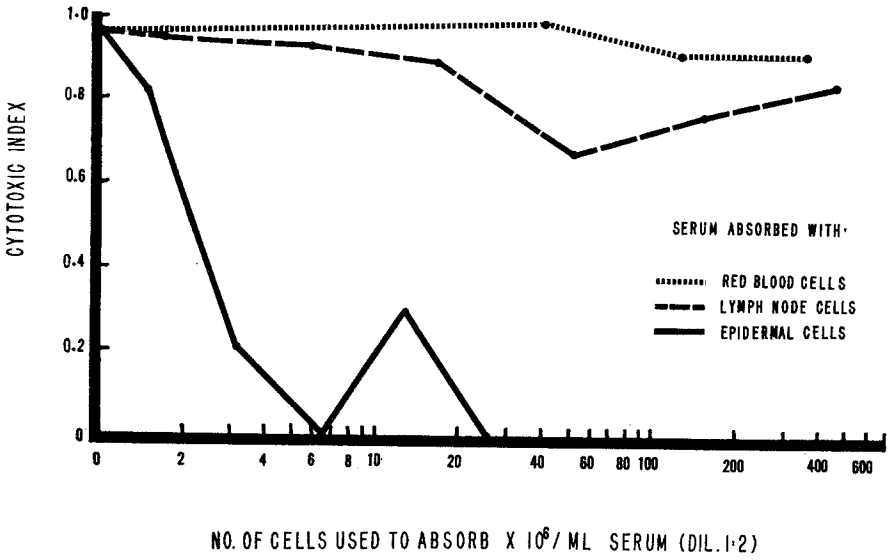

FIG. 7. Absorption of autotoxicity with various autologous cells. (Serum absorbed for 20 hours at 3°C was tested with autologous epidermal cells.)

0.14; liver cells, 0.28; and epidermal cells, 0.89. Erythrocytes and thymic cells did not fix any autologous complement.

Absorption of Toxicity.—Fresh rabbit sera absorbed with autologous erythrocytes, lymph node cells, and epidermal cells for 20 hours at 3°C were subsequently tested with autologous epidermal cells. From Fig. 7, it can be seen that whereas both erythrocytes and lymph node cells did not absorb toxicity, malpighian cells were effective at relatively low concentrations. Epidermal pulp (epidermis left after scraping away the basal layer), also absorbed activity upon 1 hour's incubation at 37°C if the packed pulp volume was ½ to ⅕ the volume of serum. With respect to duration of absorption, tests of sera following incubation with ½ volume of pulp for 10, 20, 30, and 60 minutes, resulted in

cytotoxic indices of 0.8, 0.7, 0.6, and 0.1, respectively. Both cornified buccal and vaginal cells were capable of removing the reactivity of serum at roughly similar concentrations.

Individual Specificity.—Normal rabbit serum, besides being toxic to autologous cells, was also lethal to malpighian cells of another rabbit (mean C.I. = 0.89 in 6 animals). Moreover, absorption of serum with autologous epidermal cells abolished toxicity to autologous as well as homologous cells (Table I).

Effect of Enzymes.—To test the possibility that serum plasmin was responsible for the killing of epidermal cells, EACA, an inhibitor of plasminogen activation (10), was added to fresh serum. Concentrations as high as 0.6 M did not inhibit the autotoxicity of serum. Bovine fibrinolysin was, however, toxic at

TABLE I

Specificity of Toxicity Viability of Epidermal Cells in Autologous and Homologous Serum

Epidermal cells of rabbit No.	Serum dilution	Cytotoxic index* of cells incubated 2 hours at 37°C in:			
		Serum of rabbit 928		Serum of rabbit 757	
		Unabsorbed	Absorbed with 928 epidermis	Unabsorbed	Absorbed with 757 epidermis
928	1:2	0.95	0.14	0.91	−0.05
	1:8	0.78	−0.27	0.93	0.08
757	1:2	0.94	0.41	0.79	0.37
	1:8	0.81	0.25	0.91	0.25

* C.I. (cytotoxic index) $= \frac{C - E}{C}$; C = per cent viable cells in control serum, and E = per cent viable cells in experimental serum.

concentrations greater than 1.25 per cent. The role of proteolytic enzymes was explored further with soybean trypsin inhibitor. Final concentrations of 7.5 per cent or greater were necessary to abolish the activity of fresh undiluted serum. The inhibitory effect of *p*-chloromercuribenzoate could not be determined since levels of 1.2×10^{-4} M were lethal to epidermal cells. At a concentration of 6.2×10^{-5} M it was no longer noxious, but cytotoxicity of 1:4 fresh serum was not inhibited. Lysozyme levels, tested by the method of Kirby (11), were unrelated to the varying degree of serum toxicity produced by absorption with graded quantities of inulin.

Effect of Clotting, Histamine, and Serotonin.—Plasma serum was prepared by taking blood without anticoagulants into chilled or paraffin-coated tubes, centrifuging, and allowing the separated plasma to clot (12). Such serum, obtained without allowing the clotting process to act upon the formed elements

of blood, still retained its activity (mean C.I. of four samples=0.88). The toxicity of histamine and serotonin was investigated since it has been shown that large quantities of both substances are found in rabbit serum. Whereas rabbit serum contains as much as 4 to 5 μg/ml of histamine (13), 680 μg/ml of exogenously added histamine phosphate was completely innocuous to malpighian cells. Serotonin, present normally at concentrations of 4.5 μg/ml (14) was only mildly toxic at 780 μg/ml and completely lethal only at 50,000 μg/ml.

Effect of Trypsin Upon Cells.—Various types of cells from a rabbit were

TABLE II

The Effect of Pre-Incubation in Trypsin upon Viability of Cells in Fresh Autologous Serum

Type of cell	Experiment No.	Cytotoxic index* after 2 hours' incubation in autologous serum			
		Duration of initial exposure of cells to 0.25 per cent trypsin (in minutes)			
		0	10	30	60
Polymorphonuclear leukocytes	1	0.17	0.26	0.13	
	2	0.03	0.16	0.31	0.28
	3			0.21	
Thoracic duct lymphocytes	1	−0.02	0.36	0.51	
	2	−0.03	0.13	0.10	0.02
Lymph node cells	1	0.03	0.11	0.33	
	2	−0.24	0.06	0.07	−0.02
	3			0.44	
Epidermal cells	1		0.68	0.85	0.80

* See Table I.

incubated at 37°C in 0.25 per cent trypsin for 10 to 60 minutes as noted in Table II. Upon washing in BSS and incubation for 2 hours in fresh autologous serum, cell viability was assessed with eosin dye. Pretreatment with trypsin resulted in a slightly heightened mortality of polymorphs, lymphocytes, and lymph node cells which were incubated in fresh serum as compared to the mortality of cells incubated in heated serum. However, this mortality was low when contrasted with the high percentage of malpighian cells which were killed after trypsinization for as short a period as 10 minutes. It should also be noted that polymorphs and lymphocytes in the form of single cell suspensions, were exposed more directly to trypsin than malpighian cells which were enclosed by the dermis and the cuticular layer of the epidermis during trypsinization. Attempts to circumvent the use of enzymes to procure basal cells of the epi-

dermis were uniformly unsuccessful. *Viable* cells could not be isolated by light scraping of skin, lens, cornea, iris, trachea, vaginal lining, buccal surfaces, or amniotic membranes. Although tryptic digestion could not be completely dissociated from the susceptibility of malpighian cells, it is of some significance that testicular cells, obtained without trypsinization, were killed by fresh autologous serum (mean C.I.=0.9 in 4 rats). Other untreated cells such as polymorphs and lymphocytes were not killed by serum (Table II).

Attempts to Induce "Tolerance."—Newborn rats injected intravenously with pooled epidermal cells from 4 adult rats were not "tolerant," or non-reactive to their own epidermal cells when tested 3 to 4 weeks later (Table III). Although non-inbred rats were used, some suppression of toxicity was expected since the autotoxic phenomenon was shown to be non-individual-specific.

TABLE III

Toxicity of Rat Sera to Autologous Epidermal Cells: The Effect of Age and Pretreatment at Birth

Previous treatment	Age of rats	No. of animals tested	Mean C.I.*	Range of C.I.
None	1 to 2 days	12	0.18	−0.47 to 0.61
None	3 to 4 wks.	6	0.61	0.42 to 0.71
200,000 to 400,000 epidermal cells injected iv at birth	3 to 4 wks.	11	0.67	0.24 to 0.93
None	3 mos. or older	5	0.75	0.44 to 0.94

* See Table I.

Table III serves further to illustrate the development of the autotoxic factor which was not present in newborn rats, but was present at almost adult levels in 3- to 4-week-old rats.

Effect of Antigen-Antibody Complexes and Ultrafiltration.—Soluble complexes of BSA–anti-BSA prepared at 1, 2, 3, and 6 times antigen excess were non-toxic when added in equal volumes to either absorbed serum or heated serum. When serum was ultrafiltrated through a dialysis membrane by centrifugation, activity was not present in the filtrate, but was retained by the undialyzable fraction.

DISCUSSION

The surprising result which we have been concerned with here is that epidermal cells were killed by the animal's *own* serum *in vitro*. This effect probably cannot be attributed to a pathological condition in the animals tested, since all sera of 43 "normal" rabbits were toxic to 60 to 100 per cent of autologous cells. It may be argued that the presumption of cell death by uptake of eosin dye (6, 15) is unjustified. That is, the cells may have become merely more

permeable to the dye upon pre-exposure to fresh serum. To meet this objection, measurements of cell respiration by conventional Warburg manometry and tetrazolium salts has shown that respiration was halted by fresh autologous serum but not by complement-depleted serum (data to be published).

That *heterologous* cells may be killed by fresh normal serum has been shown repeatedly (16, 17). Toxicity of heterologous serum to skin explants is mentioned by Puck *et al.*, who recommend the use of fetal agammaglobulinemic serum for the cultivation of skin cells (18). Such lethal effects of autologous and homologous sera may not have been detected previously in tissue cultures of whole skin (4) since the basal cells of the epidermis may be "protected" by the cuticle and dermis. In cultures of epidermal cell sheets (19) toxicity probably did not result because the ratio of cells to serum was, in all likelihood, higher than the presently found optimum of 1 million cells per ml of serum.

The following observations suggest that the autotoxicity of serum may occur *in vivo* and is not merely a product of the *in vitro* conditions. Both lightly heparinized plasma and plasma serum (serum obtained from plasma without anticoagulants) were active, indicating that clotting does not release toxins from formed blood elements. Although of necessity, epidermal cells were obtained by trypsinization, several experiments suggest that trypsin was not directly responsible for the death of cells. First, trypsinized epidermal cells were *not* killed by 2 hours' incubation in absorbed or complement-depleted serum. Secondly, trypsin in the concentrations used did not alter thoracic duct lymphocytes, lymph node cells, or polymorphs since these cells were not significantly affected by subsequent incubation in fresh serum. Third, autotoxicity could be absorbed by untrypsinized epidermal cells obtained from the vagina or the buccal cavity. It is also relevant that testicular cells which can be isolated without trypsin were also killed by fresh autologous serum. However, the possibility that some small change in the cells was produced by trypsinization could not be ruled out, particularly since we were unable to isolate viable epidermal cells without the aid of enzymic digestion. The autohemagglutinins demonstrable in normal human serum against trypsinized erythrocytes (20, 21) seem to be unrelated to the toxic factors since they act independently of complement (21) and apparently do not cause autohemolysis in the presence of complement. Versene was shown to be harmless in the concentrations used, since later tests in which versene was completely eliminated led to identical results.

With regard to the factors responsible for autotoxicity, it is plausible that enzymes present either in the serum or in the epidermal cells themselves cause death of the cells. The observation that the number of dead cells increased in proportion to the number of cells used per unit volume of serum indicates that some enzymic process may be involved. Among the many enzymes present in serum, proteases at least do not seem to be involved since EACA (10) and

trypsin inhibitor (22) did not inactivate serum. The proteinases in skin which have been characterized thus far are unlikely to produce toxicity since they are present in high levels in rat skin but are apparently absent in rabbit skin (23). In any event, it remains to be seen whether various other enzymes present in serum or cells could cause death of the malpighian cells.

On the basis of recent findings that non-specific antigen-antibody complexes can cause a local cutaneous reaction (24), anaphylaxis (25), and glomerular damage (26), it might be postulated that these complexes, normally present in the serum, may kill epidermal cells. However, soluble BSA–anti-BSA complexes made in antigen excess were inactive when added together with complement to epidermal cells. Also, histamine and serotonin were innocuous in concentrations normally found in serum.

The hypothesis that epidermal cells were killed by auto-antibodies is supported by two principal series of experiments. First, it appears certain from the data presented that complement-like components are incriminated. Thus, autologous serum activity was destroyed by heating serum at 52°C for 10 minutes and by treatment of serum with zymosan, ammonium hydroxide, and BSA–anti-BSA precipitate. Sera depleted of C'_3 and sera depleted of C'_4 regained their activity upon recombination. Nevertheless, it is clear that complement by itself was not lethal since newborn rat sera which contained high levels of complement, were not toxic. The fact that mouse sera, commonly known to be low in C′ activity, were inactive is consistent with the implication of complement; although mouse sera may be exceptional for various other reasons. Though complement could conceivably act with other substances, such as proteases (22), the requirement of complement components and the "fixation" of complement can be most readily explained by the activity of complement with antibody. Perhaps the strongest evidence that antibody is involved can be found in the specificity of the phenomenon. That is, epidermal cells and testicular cells were killed by autologous serum, but lymphocytes and polymorphs were unaffected. Moreover, cytotoxicity could be absorbed by malpighian cells, though not by lymph node cells or erythrocytes. Absorption, however, was not individual-specific in that homologous epidermal cells also absorbed toxicity. This type of affinity is similar to that of most auto-antibodies which are tissue-specific though not individual-specific (27, 28). Perhaps some admissible support for the existence of natural auto-antibodies to epidermal cells can be found in the observation that epidermal cells injected intravenously often lead to immediate death, suggesting anaphylaxis (29, 30). It should be noted however, that to date, we have been unable to elute antibody from cells or to demonstrate that serum depleted of complement can be reactivated by absorbed serum or by newborn rat serum. In addition, tolerance to epidermal cells, in the dosages employed, could not be induced in newborn rats.

If the autotoxic phenomenon herein described is not one which is solely

dependent on the *in vitro* conditions, but is indicative of the situation in the living organism, several interesting speculations might be made. In the body under normal conditions, either the basement membrane or the capillary or lymphatic walls may act as a separating barrier between the plasma and epidermis. Various pathological lesions which lead to death and necrosis of the epidermis might then be attributed to a breakdown in this barrier. As an example, the necrosis of the Arthus reaction can be produced by the local increase in permeability caused by an antigen-antibody complex (24) followed by the release of the autotoxins from the circulation. Destruction of the epidermis in the homograft reaction may conceivably be produced by a reaction upon the barrier followed by damage of cells by autotoxic factors. It might further be speculated that if a plasma-epidermis barrier exists, an animal may not become tolerant of its own epidermal cells according to the Medawar-Burnet hypothesis (31). In later life, skin injuries may serve to immunize the animal against its own epidermal cells. That auto-immunization to skin may occur has been suggested (32, 33), though one direct attempt has been negative (34).

SUMMARY

Sera and plasma from normal rats and rabbits were shown to be extremely toxic *in vitro* to *autologous* epidermal cells. On the other hand, mouse sera and newborn rat sera were innocuous to autologous epidermal cells. Viability of cells was assessed by the method of eosin dye exclusion upon 2 hour incubation at 37°C. Testicular cells were also killed by autologous sera, but polymorphonuclear leukocytes, lymphocytes, and lymph node cells were not affected.

Autotoxicity of sera could be destroyed by the depletion of complement components with an antigen-antibody precipitate, heat, zymosan, and NH_3. Moreover, activity of sera could be absorbed out by epidermal cells, though not by lymph node cells or erythrocytes. Such absorption of toxicity was not individual-specific since homologous epidermal cells also absorbed toxicity, and in addition, were killed by fresh normal serum. Enzyme inhibitors such as soybean trypsin inhibitor and ϵ-amino-*n*-caproic acid did not affect the activity of fresh autologous serum.

It is suggested that a natural barrier exists between the basal cells of the epidermis and the plasma which prevents the autodestructive process under normal conditions. Any injury to this barrier may than lead to necrosis and death of the epidermis as seen in various pathological conditions.

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