DELAYED HYPERSENSITIVITY

I. Effect of In Vitro Exposure of Cells to Antigen upon Leukocytic Transfer of Delayed Hypersensitivity*

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The mechanism of delayed hypersensitivity is poorly understood due in large part to limited techniques for in vitro study. With the exception of the human species, live leukocytes obtained from peripheral blood, lymph nodes, spleen, or peritoneal washings have been required to transfer delayed hypersensitivity from a sensitized donor to a normal recipient (1). Study of direct interaction between sensitized donor cells and antigen, in the recipient's skin, is hampered by the finding that most of the leukocytes at the site of a delayed reaction are from the recipient and not the donor (2). The present studies were designed to investigate the interaction of donor cells and antigens in vitro, and to study the effect of antigen exposure upon the ability of sensitized donor cells to transfer delayed sensitivity. The results indicate that exposure to certain antigens reduced or ablated the transfer of delayed hypersensitivity, and that this interference was related to the degree to which antigens remained associated with cells after exposure in vitro. Evidence is presented that size of the antigen was more important than either degree of conjugation, or charge, in determining cell association of antigens.

Materials and Methods

Reagents.—Picryl chloride was obtained from Eastman Organic Chemicals, Rochester, N.Y. Acetic-1-^{14}C-anhydride, obtained from Nuclear-Chicago Corp., Des Plaines, Ill., was prepared to contain 2 μc/ml. Radioactive iodine (^{131}I) was obtained from E. R. Squibb and Sons, New York, carrier-free, without reducing agent, prepared to contain 1 mc/ml. Dextran, molecular weight 100,000–200,000, was obtained from Nutritional Biochemical Corporation, Cleveland, Ohio. Parachlorobenzoyl chloride (PCBC) was obtained from Matheson, Coleman, and Bell, Cincinnati, Ohio.

Antigens.—Guinea pig serum albumin (GPA) was prepared by starch block electrophoresis (3), and purity was established by immunoelectrophoresis. Conjugates of varying mounts of picryl groups attached to GPA were all prepared by the same method: GPA was dissolved

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in saline to a concentration of 1% and the pH adjusted to 9.5–9.8 with 5% sodium carbonate. Varying concentrations of picryl chloride were diluted in 1,4-dioxane and added dropwise to the GPA solutions in order to obtain preparations with different average numbers of hapten groups per molecule. The concentration of dioxane never exceeded 5% by volume. Solutions were allowed to react with constant stirring for 2 hr; unreacted hapten was separated from the conjugate by Sephadex G-25 gel filtration. The average number of hapten groups per molecule of GPA conjugate was calculated from its optical density at 347.5 mμ using a molar extinction coefficient of 15,400. Protein concentrations were calculated from micro-Kjeldahl nitrogen determinations. Various conjugates of picryl guinea pig albumin (PicGPA) were prepared with 3.8 to 48.0 picryl groups per molecule of protein. Conjugates containing 44–48 picryl groups were designated PicGPA-hi.

Acetylation of Antigen.—PicGPA with 6.0 hapten groups per mole, designated PicGPA-lo, was acetylated according to the method described by Fraenkel-Conrat et al. (4); 1.0 ml containing 2 μc of acetic-1-^{14}C-anhydride was added dropwise to 24 mg of PicGPA-lo dissolved in 8.0 ml of saturated sodium acetate solution. Addition of N NaOH to the mixture maintained pH at 8.0. After 2 hr, the acetic anhydride was separated from the acetylated protein (PicGPA-lo-acet) by gel filtration through Sephadex G-25. The labeled antigen was counted in a liquid scintillation counter (Nuclear-Chicago Corp., Des Plaines, Ill.) and 33 acyl groups were calculated per mole of protein.

Immunization.—Male and female albino guinea pigs of the Hartley strain, weighing 400–500 g, were immunized with antigens emulsified in complete Freund's adjuvant (Difco Labs, Inc., Detroit, Mich.). Each animal received 100 μg of antigen distributed in the four foot pads in a volume of 0.1 ml per foot pad.

Preparation of Leukocyte Suspensions, Antigen Exposure, and Transfer.—Two sources of leukocytes were used in the experiments to be described; peripheral blood and peritoneal exudates. Peripheral blood leukocytes were obtained on the 7th day after immunization; donors were injected intravenously with 0.6 ml of sodium heparin (1,000 USP units/ml of Liquaemin, Organon, Inc., West Orange, N.J.), lightly anesthetized with ether, and exsanguinated by cardiac puncture. Two volumes of 3% dextran and saline were added to one volume of freshly drawn blood. The tubes were gently inverted and then placed on a slant to allow erythrocytes to settle. After 20–30 min, leukocyte-rich supernates were removed and centrifuged at 1200 *g* for 12 min at 4°C. Leukocytes were washed without heparin, resuspended in 199 culture medium (Baltimore Biological Laboratory, Baltimore, Md.), and divided into portions for exposure to homologous or heterologous antigen at a final concentration of 100 μg/ml; the mixture of antigen and cells was incubated at room temperature for 20–30 min, then washed 3 times in 199 medium. Before intravenous injection into recipients, cells were counted and volumes adjusted so that each recipient received approximately 1.5×10^8 cells.

Peritoneal exudates were also harvested on the 7th day after immunization. Donor animals were injected intraperitoneally with 5 ml of sterile proteose-peptone broth (Difco Labs) on the 5th day after immunization. At harvest, on the 7th day, donors were exsanguinated and the peritoneal cavity repeatedly washed with 199 solution. Recipients of peritoneal exudate leukocytes received 1.5×10^8 cells intravenously. Results were always similar when different sources of leukocytes were compared; in the early stages of this study, peripheral blood was a common source of leukocytes, and in the later stages, peritoneal exudates.

Skin Tests.—Recipients weighing 250–300 mg were challenged intradermally in a shaved flank with 30 μg of antigen in 0.1 ml of saline immediately after transfer. Delayed reactions appeared after 6 hr and reached maximum intensities at 18–24 hr. Reactions were measured and recorded at 24 hr in the manner previously described (5, 6). Area less than 5×5 mm were read as negative. Contact sensitivity to PCBC was transferred as described above; recipients were challenged with application of 0.1 ml of 2% antigen in an acetone–olive oil vehicle (4:1). At 24 hr reactions were scored 1+–4+ on the basis of redness and induration (7).

RESULTS

Effect of Exposure of Cells to Antigen.—Peripheral blood leukocytes from donors sensitized to PicGPA-hi were exposed in vitro to PicGPA-hi. 11 of 28 animals developed delayed sensitivity reactions at the site of challenge as shown in Table I. The average size of positive skin tests was 4 × 6 mm. In contrast, 32 of 37 control animals receiving leukocytes from donors sensitized to PicGPA-hi reacted positively when challenged with 30 μg of the homologous antigen as shown in Table I. The average size of positive skin test was 8 × 10 mm, clearly demonstrating that exposure of leukocytes from donors sensitized to PicGPA-hi affected the ability of donor cells to transfer delayed sensitivity.

TABLE I

Effect Upon the Transfer Reaction of Exposure of Sensitized Cells to Heavily Conjugated (PicGPA-hi) and lightly Conjugated (PicGPA-lo) Antigens

Donors immunized with	Leukocytes exposed to 100 μg/ml	Amount antigen transferred per recipient	Amount antigen injected i.v.	No. successful transfers / No. recipients	Successful transfers	Average size positive skin tests
		μg	*μg*		*%*	*mm*
PicGPA-hi	PicGPA-hi	90	—	11/28	39	4 × 6
PicGPA-hi	HSA	—	—	32/37	86.4	8 × 10
PicGPA-lo	PicGPA-lo	1.4	—	45/61	73.7	6 × 8
PicGPA-lo	HSA	—	—	45/57	78.9	6 × 7
PicGPA-lo	PicGPA-lo	1.5	90	7/8	87.5	6 × 6
PCBC	PicGPA-hi	93	—	8/8	100	3+

When peripheral blood leukocytes obtained from donors sensitized to PicGPA-lo were exposed in vitro to the homologous antigen, 45 of 61 recipients reacted at the site of challenge as indicated in Table I. The average size of positive skin tests in these experiments was 6 × 8 mm. In the control group, 45 of 57 recipients developed delayed reactions comparable in size to those observed in the experimental group; homologous antigen exposure of leukocytes from donors sensitized to lightly conjugated protein did not cause ablation or reduction of the delayed response, a result clearly different than that obtained with heavily conjugated antigen.

Effect of Exposure of Cells to a Heterologous Antigen.—To determine whether "desensitization" was due to a toxic effect of the PicGPA-hi antigen, a heterologous system was used, as indicated in Table I. Peripheral blood leukocytes obtained from donors sensitized to parachlorobenzoyl chloride (PCBC) were exposed in vitro to PicGPA-hi. Recipients developed good contact reactions 24 hr after challenge with 2% PCBC, indicating that ablation of the delayed response in recipients of cells from homologously sensitized donors was not due to a toxic, nonspecific effect of antigen upon donor leukocytes.

Cell Association of Antigen.—The amount of antigen which remained associated with cells after antigen exposure was measured by labeling both the lightly conjugated and the heavily conjugated protein with ^{131}I. Peripheral blood leukocytes from donors sensitized to PicGPA-lo were exposed in vitro to ^{131}I-labeled PicGPA-lo. After three washes, radioactivity of the cells was counted. Less than 2 μg of antigen remained associated with the cells as shown in Table I. In contrast, after three washes approximately 90 μg of PicGPA-hi antigen remained associated with the peripheral blood leukocytes from donors sensitized to PicGPA-hi which had been exposed to ^{131}I-labeled PicGPA-hi in vitro. To determine whether antigen which remained associated with leukocytes caused "desensitization" of the recipient guinea pig, leukocytes obtained from donors sensitized to PicGPA-lo were exposed to PicGPA-lo, washed three times and injected intravenously into the right hind foot of the recipient. Simultaneously 90 μg of PicGPA-lo was injected intravenously into the left foot. Results in Table I show that 87.5% (7/8) of the recipients developed delayed sensitivity at the site of challenge, demonstrating that the antigen transferred did not affect the recipient's ability to become passively sensitized.

Specificity of Antigen Uptake.—Peripheral blood leukocytes obtained from donors sensitized to PCBC were exposed in vitro to 100 μg/ml of ^{131}I-labeled PicGPA-hi. After three washes, approximately 90 μg of antigen remained associated with cells. This amount of antigen had no effect on the elicitation of the delayed response in recipients; all eight animals reacted positively to contact challenge with 2% PCBC as indicated in Table I. While antigen uptake is demonstrated to be nonspecific, the effect upon homologously sensitized donor cells is specific.

Characterization of the Various Protein Conjugates.—Among the properties of an antigen which affect its ability to become associated with cells is its charge (7). Conjugates with 40 haptenic groups per mole have greater net negative charges than conjugates having 6 haptenic groups per mole. In order to increase the negative charge, the lightly conjugated antigen, PicGPA-lo, was acetylated. Immunoelectrophoretic patterns of the various protein conjugates are shown in Fig. 1: In the top well of each slide PicGPA-lo was placed; in the bottom well, top slide, PicGPA-hi; in the bottom well, bottom slide, acetylated PicGPA-lo (PicGPA-lo-acet). After electrophoresis, a center trough was cut into each slide and filled with rabbit anti-picryl human serum albumin (HSA). PicGPA-hi, because of its higher negative charge, is shown to migrate faster than PicGPA-lo. However, the addition of acyl groups to the PicGPA-lo resulted in a migration comparable to that of the highly conjugated albumin. Two precipitin lines appeared in the PicGPA-lo-acet immunoelectrophoretic pattern, an observation which has been observed and investigated previously (8).

The biological effect of charge on desensitization was determined by in vitro

exposure of 100 μg/ml of PicGPA-lo-acet to leukocytes obtained from donors sensitized to PicGPA-lo-acet. As indicated in Table II, 92.8% of transfers in 14 controls and 100% of transfers in 13 experimental recipients were successful. Thus, charge alone was not responsible for effecting desensitization.

Effect of Antigen Size on Sensitized Cells.—The substitution of picryl groups on GPA favors molecular aggregation (9). Accordingly, sedimentation studies of GPA and the various protein conjugates were performed upon 1% protein solutions in the Spinco model E ultracentrifuge. Fig. 2 illustrates the sedimenta-

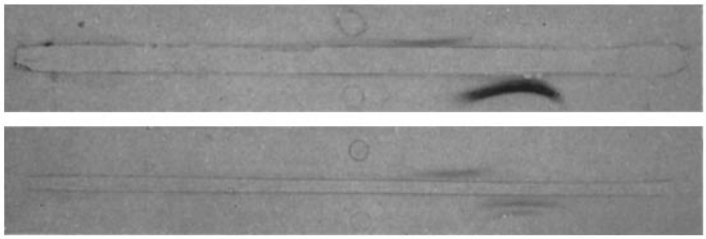

FIG. 1. Immunoelectrophoresis of PicGPA-lo (top well, each slide), PicGPA-hi (bottom well, top slide) and PicGPA-lo-acet (bottom well, bottom slide) vs. rabbit anti-PicHSA (both troughs).

TABLE II

Effect of Acetylation of Lightly Conjugated PicGPA Antigen upon Desensitization of Donor Leukocytes

Donors immunized with	Leukocytes exposed to 100 μg/ml	No. successful transfers / No. recipients	Successful transfers	Average size positive skin tests
			%	*mm*
PicGPA-lo-acet	HSA	13/14	92.8	8 × 8
PicGPA-lo-acet	PicGPA-lo-acet	13/13	100	8 × 9

tion patterns obtained at varying speeds and time periods. No significant difference was noted in the sedimentation rates of native GPA, PicGPA-lo, and PicGPA-lo-acet antigens. However, the sedimentation pattern of PicGPA-hi differs markedly from those patterns produced by GPA, PicGPA-lo, and PicGPA-lo-acet. After 13 min at a speed of 29,000 rpm, protein had reached the base line with a slope characteristic of heterogeneity.

Attempts to aggregate PicGPA-lo with heat so that the ratio of substituted picryl groups per protein molecule remained approximately 3 to 6 were uniformly unsuccessful. GPA was therefore aggregated by the method of Iio and Wagner (10) as modified in this laboratory (11). Aggregated albumin was then picrylated as described above so that the ratio of picryl groups to protein nitro-

gen was the same as in PicGPA-lo, i.e., approximately 1.0. Lightly conjugated aggregated albumin (PicGPA-agg-lo) was labeled with ^{131}I to compare it with PicGPA-lo and PicGPA-hi with respect to cellular uptake. This comparison was obtained by measuring clearances of the respective antigens by the reticulo-endothelial system of mice (7). Mice were injected with trace-labeled antigens in concentration of 1 mg/100 g of mouse and bled from the retro-orbital sinus

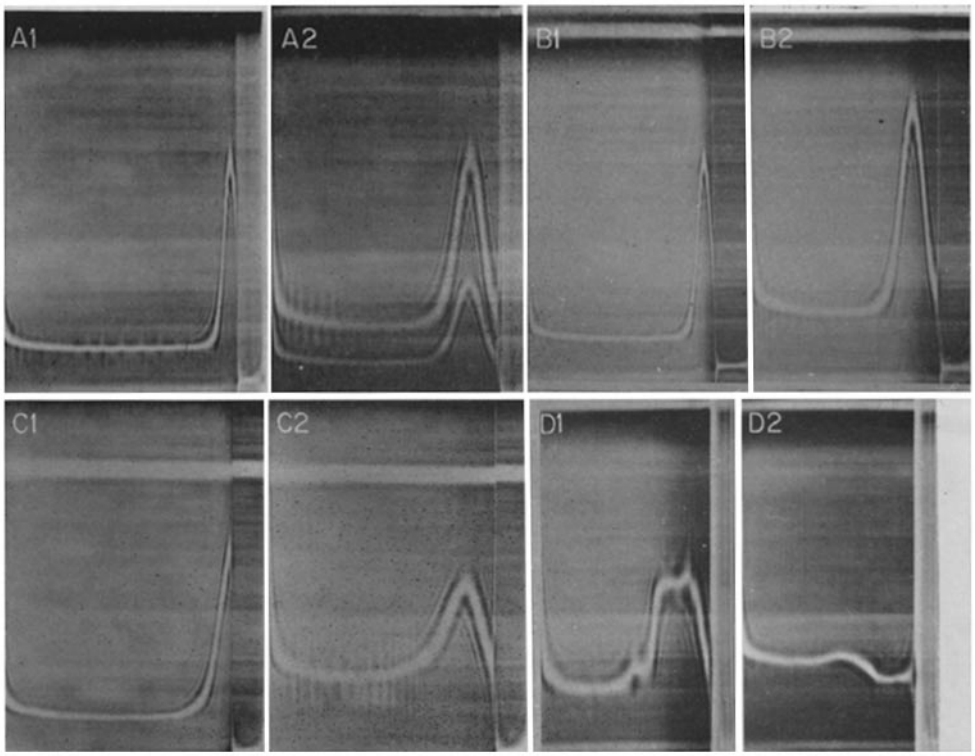

FIG. 2. Ultracentrifugal analysis of GPA, PicGPA-lo, PicGPA-lo-acet, and PicGPA-hi.

A. GPA: Frame 1, sedimentation pattern at speed of 39,460 rpm and angle of 50°, 17 min after start of run. Frame 2, sedimentation pattern at speed of 39,460 rpm and angles of 50° and 30°, 60 min after start of run.

B. PicGPA-lo: Frame 1, sedimentation pattern at speed of 39,460 rpm and angle of 50°, 27 min after start of run. Frame 2, sedimentation pattern at speed of 39,460 rpm and angle of 50°, 60 min after start of run.

C. PicGPA-lo-acet (33 groups per mole): Frame 1, sedimentation pattern at speed of 39,460 rpm and angle of 50°, 28 min after start of run. Frame 2, sedimentation pattern at speed of 39,460 rpm and angle of 30°, 67 min after start of run.

D. PicGPA-hi: Frame 1, sedimentation pattern at speed of 29,000 rpm and angle of 20°, 7 min after start of run. Frame 2, sedimentaton pattern at speed of 39,400 rpm and angle of 30°, 13 min after start of run.

at designated time intervals. Counts per minute were plotted vs. time on semilog paper as shown in Fig. 3. The slope of clearance increased so that PicGPA-lo < PicGPA-hi < PicGPA-agg-lo.

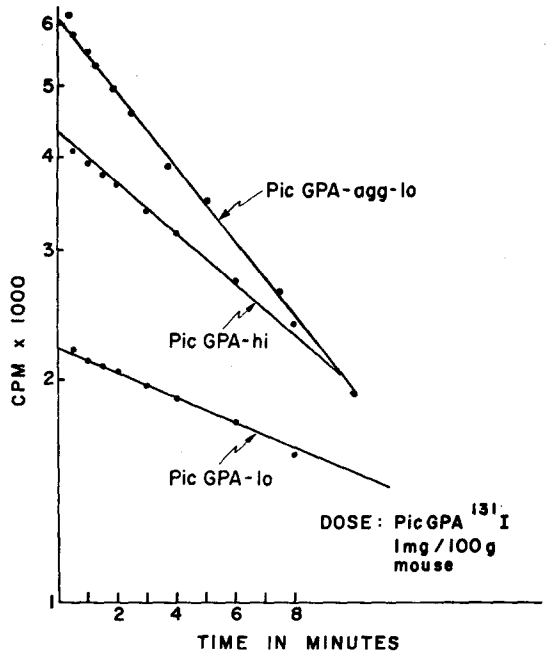

FIG. 3. Blood clearence of picryl conjugates in mice. Three mice were used to test each antigen. Radioactivity was counted long enough to ensure a counting error of less than 3%.

TABLE III

Effect of Aggregation of Antigen upon Desensitization of Donor Leukocytes

Donors immunized with	Leukocytes exposed to 100 μg/ml	No. successful transfers / No. recipients	Average size of reaction
			mm
PicGPA-agg-lo	HSA	11/12	10 × 12
PicGPD-agg-lo	PicGPA-agg-lo	3/12	4 × 5
PCBC	PicGPA-agg-lo	8/10	3+

Donor animals were immunized with 100 μg of PicGPA-agg-lo, and cells obtained on the 7th day were exposed to the immunizing antigen prior to transfer in the manner described above. As shown in Table III, only 3 of 12 recipients had minimally positive transfer reactions while 11 of 12 recipients of the same pool of cells exposed to HSA were positive with reactions averaging

12 mm in diameter. Exposure of cells from PCBC-sensitized animals to PicGPA-agg-lo failed to affect the ability of cells to transfer the contact reaction.

DISCUSSION

Peritoneal inflammatory cells from sensitized guinea pigs were unable to transfer delayed sensitivity to normal recipients after a short exposure to antigen in vitro. In many respects these experiments are similar to those performed by Lawrence and Pappenheimer (12). They exposed cells, in vitro, from humans sensitive to two different antigens, to one of the antigens, and observed the inability of the cells to then transfer delayed hypersensitivity to the exposed antigen. However, ability to transfer sensitivity to the second antigen was retained. In the present studies, using the same hapten and carrier combination in different proportions, two antigens were found which differed in their ability to affect homologously sensitized cells by exposure in vitro. The highly conjugated antigen clearly affected the ability of homologous cells to transfer sensitivity while a lightly conjugated antigen did not. Initially, charge was investigated as a source of differences to explain the observations: When the charges were equalized by the addition of acyl groups to the lightly conjugated antigen, no change in ability of the lightly conjugated antigen to desensitize cells was observed. In ultracentrifugal studies, highly conjugated picryl GPA was shown to be self-aggregating and contained many high molecular weight aggregates. When lightly conjugated aggregated albumin was prepared, it became clear that this antigen was also capable of affecting the ability of homologously sensitized cells to transfer delayed sensitivity.

The influence of size upon the antigenic properties of a protein has been much appreciated (7, 13). In the case of bovine gamma globulin, several investigators have shown that aggregated gamma globulin is a potent antigen when injected into animals, whereas unaggregated globulin produced not sensitization, but tolerance (14–16). With the exquisitely sensitive flagellar antigen used by Nossal et al., the immune response differed depending on whether aggregated flagellar antigen or monomeric forms were used (17).

A short antigen exposure of 30 min was apparently long enough for heavily conjugated antigen and the aggregated lightly conjugated PicGPA to become associated with cells. Whether this association was upon the surface or within the cell is not clear from these data, but the quality of becoming associated with cells seems to bear a relationship to the ability of the antigen to desensitize cells. While the mechanism of delayed hypersensitivity with special reference to the moiety which recognizes antigen upon or within the cell is unknown, two possibilities are evident: The first would involve the production of a small amount of a highly unusual antibody at the surface of the cell capable of recognizing antigen at that site. If desensitization were a function of occupying these specific antibody sites on the surface of sensitized cells, then the requirements

for antigen would involve only the number of molecules present, since the number of cells remains the same and the association constant would be a quality of the "delayed hypersensitivity antibody." In the experiments cited above, simple interaction of soluble antigen with the presumed antibody molecules on exposed cell receptor sites could not explain desensitization in the case of the highly conjugated antigen and the failure to desensitize with lightly conjugated antigens. The same weight concentration was used for each attempted desensitization. With the heavily conjugated aggregated antigen, a given weight would result in a lower molar concentration than would the smaller, lightly conjugated PicGPA-lo. Notwithstanding its higher molar concentration, the lightly conjugated antigen failed to desensitize cells in vitro under the conditions of the experiment. These data suggest that factors other than strict antigen-antibody interactions occurred on the surface of the cell, or that factors important in the recognition and initial phases of the delayed sensitive reaction are not on the surface of the cell. An alternate explanation proposes that the cell interacts with antigen in a biologic way, i.e. by phagocytosis, pinocytosis, or a similar process. Only as a secondary event would recognition of the antigen occur by the specifically sensitized cell or a product of this cell and thereby effect desensitization. This postulate is consistent with the data: Antigen capable of causing desensitization, PicGPA-hi, was taken up in equal amount by both homologously and heterologously sensitized cells, but desensitization was a specific event.

Peritoneal exudate cells and lymphocytes prepared from such exudates, when exposed to antigen, have recently been reported to release a mediator capable of inhibiting migration of normal macrophages from capillary tubes (18). Duration of exposure was several hours. If desensitization is accompanied by release of the mediator which inhibits macrophage migration, supernates of reaction mixtures containing PicGPA-hi should inhibit migration of normal cells from capillary tubes, while supernates of reaction mixtures containing PicGPA-lo should not. The in vitro system described above permits such studies, now in progress, which may relate the afferent or recognition limb with the efferent or effector arm of the delayed hypersensitivity reaction.

The effect of antigen exposure upon the sensitized cell is uncertain. Desensitization may cause cells to release "mediator" or otherwise become temporarily altered so as to become unable to convey sensitivity to a normal recipient. Alternatively, the exposure and uptake by sensitized cells could result in injury and cell death so that the 1.5×10^8 cells were not a mixture of sensitized and normal cells, but only normal cells with respect to the antigen involved. If delayed sensitivity returned to the recipient of exposed cells as it does in actively sensitized animals exposed to antigen in vivo (19), the former possibility would be favored. Unfortunately the presence of antigen infused with the sensitized cells makes it necessary for the recipient to be immunologically impaired. Experiments along these lines are now in progress.

SUMMARY

Exposure to picryl guinea pig albumin with 3–6 picryl groups per mole failed to affect the ability of peritoneal exudate or peripheral blood leukocytes from sensitized donors to transfer delayed sensitivity to normal recipients. In contrast, conjugates containing 40–48 picryl groups per mole altered the ability of exposed leukocytes to transfer delayed sensitivity. Evidence is presented that highly conjugated guinea pig albumin is self-aggregating. Lightly conjugated albumin, previously heat-aggregated, also was effective in "desensitization." The properties of antigen size, cell association of antigen after exposure, and desensitization appear to be associated.

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