

## ROLE OF ANTIGEN-PRESENTING CELLS IN THE DEVELOPMENT AND PERSISTENCE OF CONTACT HYPERSENSITIVITY\*

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Immunization of mice by intravenous injection of relatively low doses of sheep erythrocytes (SRBC)<sup>1</sup> leads to a potent but evanescent form of delayed-type hypersensitivity (DTH) (1). 4 d after immunization, the injection of the appropriate antigen into the footpad of a mouse leads to induration and swelling 24 and 48 h later. By 10–14 d after immunization, similar challenge elicits significantly weaker reactions, or no reactions at all (1). On the other hand, immunization with antigen emulsified in complete Freund's adjuvant or administered with bacilli Calmette-Guérin (BCG) results in a much longer-lived immunity of a similar type (2). The waning of the evanescent form of the DTH described above does not appear to be a result of suppressor cell activity, and thus one form of DTH is most likely mediated by an inherently short-lived T cell (1).

We now report that contact hypersensitivity reactions (CS), like DTH reactions discussed above, occur in two forms: an evanescent and a long-lived class. Skin painting with a reactive form of the immunogen leads to a long-lived form of immunity. On the other hand, we have confirmed the findings of Greene et al. (3) that subcutaneous injections of the immunogen conjugated to peritoneal exudate cells (PEC) lead to a short-lived form. Again in agreement with Greene et al. (3) we have found that intravenous injection of immunogen conjugated to PEC fails to yield any class of CS, and in fact leads to specific immunological unresponsiveness for CS induced by contact painting. However, if mice which are immunized intravenously with hapten-conjugated PEC, are pretreated with cyclophosphamide (CY), CS can then be elicited.

In contrast, intravenous injection of hapten-conjugated epidermal cells does not require CY pretreatment to induce immunity; nor does the immune state rapidly

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<sup>1</sup> Abbreviations used in this paper: BCG, bacilli Calmette-Guérin; CRBC, chicken erythrocytes; CS, contact hypersensitivity reaction(s); CY, cyclophosphamide; DTH, delayed-type hypersensitivity; HBSS, Hanks' balanced salt solution; HRP, horseradish peroxidase; MHC, major histocompatibility complex; MRBC, mouse erythrocytes; OX, oxazalone; PCL, picryl chloride; PEC, peritoneal exudate cells; PSA, trinitrobenzene sulfonic acid; SRBC, sheep erythrocytes; Thy, thymocytes; TNP, trinitrophenyl; TNP-CRBC, TNP-substituted CRBC; TNP-epidermal cells, TNP-conjugated epidermal cells; TNP-erythrocytes, TNP-conjugated erythrocytes; TNP-PEC, TNP-substituted PEC; TNP-Thy, TNP-substituted Thy.

wane. Therefore, immunization with epidermal cells conjugated with antigen induces a class of CS more like the one induced by skin painting than like the one induced by injection of antigen-conjugated PEC. Thus, not only the anatomical site where antigen is first encountered by the immune apparatus, but also the nature of the cells that present the antigen, determine whether a DTH response will ensue, as well as whether it will be evanescent or long-lasting.

### Materials and Methods

*Animals.* Inbred CBA mice of both sexes, weighing 22–25 g were used in all experiments. Only one sex was used in any one experiment. These mice and BALB/c mice were obtained from the breeding unit of the Institute of Microbiology, Cracow, Poland. Guinea pigs were purchased from a local supplier in Poland.

*Reagents.* The following reagents were used: picryl chloride (PCL) (Fluka AG, Buchs, Switzerland); trinitrobenzene sulphonic acid (PSA) and oxazolone (OX) (4-ethoxymethylene-2-phenyloxazolone) (British Drug Houses Ltd., Poole, Dorset, England); [<sup>51</sup>Cr]sodium chromate (Institute for Nuclear Research, Warsaw, Poland); and CY (VEB, Jenapharm, Democratic Republic of Germany).

#### *Cells*

**MACROPHAGES.** Peritoneal exudates were induced by i.p. injection of 3 ml of thioglycollate medium (Difco Laboratories, Detroit, Mich.) or 3 ml Marcol 52 oil (Exxon Corp., New York). The cells harvested 4 or 5 d later, respectively, were 90–95% macrophages and were termed PEC throughout.

**THYMOCYTES.** Thymuses were removed after exsanguination and were gently teased apart between the frosted ends of microscope slides into the cold Hanks' balanced salt solution (HBSS).

**EPIDERMAL CELLS.** Epidermal cells were obtained from the tail skin by trypsinization according to the method of Scheid et al. (4) with minor modifications. In one experiment, epidermal cells were separated into fractions enriched and depleted of Fc-positive cells. Epidermal cells were incubated (37°C, 45 min) with SRBC coated with mouse anti-SRBC antibody at a ratio of 10 SRBC:epidermal cells, and then were layered over Ficoll (Pharmacia Fine Chemicals, Div. of Pharmacia, Inc., Piscataway, N. J.)-sodium metrizoate (12 parts 14% Ficoll 400 + 5 parts 32.8% sodium metrizoate) and centrifuged for 30 min at 2,000 g. Cells from the overlayer and pellet were freed of erythrocytes by treatment with 0.17 M NH<sub>4</sub>Cl, labeled with trinitrophenyl (TNP), and injected intravenously into CBA mice. The pellet fraction contained 18% of the skin cells and was called Fc enriched, whereas the overlayer was called Fc depleted.

**ERYTHROCYTES.** Mouse erythrocytes (MRBC) were collected after injection of 200 U heparin i.v. per mouse. Before hapten labeling, the buffy coat was removed. Chicken erythrocytes (CRBC) were a mixture obtained from three donors. SRBC were obtained from a single animal.

*Hapten Labeling of Cells.* All cells were washed several times in HBSS before they were labeled with hapten. TNP-substituted erythrocytes were prepared as described by Rittenberg and Pratt (5) with minor modifications (6). 20 mg of PSA per 7 ml of cacodylate buffer were used to label 1 ml of packed erythrocytes. PSA in HBSS (3 mg/ml) was used to label  $5 \times 10^7$  nucleated cells for 10 min at room temperature. OX was dissolved in absolute ethanol (10 mg/ml) and mixed rapidly with 20 ml of EDTA buffer (0.05 M, pH 8.4). The solution was used immediately for cell labeling (7). 1 ml of packed MRBC was suspended in 10 ml calcium-free HBSS and mixed with 20 ml of OX solution. Nucleated cells were suspended in HBSS at  $10^7$ /ml and mixed with a double volume of OX solution diluted previously 1:1 with EDTA buffer. PEC and thymocytes were labeled for 10 min at room temperature, whereas MRBC were labeled for 30 min. After labeling, cells were washed twice with EDTA buffer (0.05 M, pH 7.5), once with HBSS, then were filtered through nylon N110 gauze, and the cell density was adjusted to the desired concentration. Viability was assessed by trypan blue dye exclusion and ranged between 70 and 85%. Generally, ~50% of the cells were recovered after hapten conjugation.

**Antibodies.** CBA mice were injected three times at weekly intervals with  $5 \times 10^8$  TNP-substituted CRBC (TNP-CRBC), and bled out 7 d after the last injection. The hemagglutination titer of the pooled sera was 1:1,024 with TNP-substituted SRBC. Guinea pigs were injected with  $10^9$  MRBC,  $10^8$  thymocytes, or  $5 \times 10^8$  TNP-CRBC i.v. at two occasions 2 wk apart and bled out 6 d later. Anti-MRBC and anti-thymocyte antibody were adsorbed (1:1) with thymocytes or MRBC, respectively, and no cross-reactions were found in hemagglutination. The hemagglutination titers of adsorbed guinea pig anti-MRBC and anti-TNP sera were 1:1,024 and 1:512, respectively. Anti-thymocyte serum diluted 1:40 killed  $10^7$  thymocytes per ml in the presence of complement. Sera were heat inactivated at  $56^\circ\text{C}$  for 30 min before being used.

**Preparation of (Fab')<sub>2</sub> Fragments.** The IgG-containing fraction of anti-TNP antibody in guinea pig immune serum was precipitated three times with 33% saturated  $\text{NH}_4\text{SO}_4$ . Pepsin digestion was performed as described elsewhere (8). The digested globulin fraction was dialyzed into 0.1 M phosphate buffer, pH 6.8, and was applied to a  $2 \times 60$ -cm Sephadex G-100 column (Pharmacia Fine Chemicals, Div. of Pharmacia, Inc.). Protein concentration in each fraction was estimated spectrophotometrically at 280 nm, and the hemagglutination titer of each fraction was determined. Fractions showing the peak values were pooled and concentrated by ultrafiltration. The hemagglutination titer of the (Fab')<sub>2</sub> preparation was 1:320.

**Coating of TNP-substituted Cells with Antibody.** TNP-labeled cells were incubated for 45 min at room temperature with antibody directed against the hapten or the cell to which the TNP was attached. As a routine procedure,  $1 \times 10^8$  cells were incubated with 1 ml of corresponding antibody diluted 1:10.

**Feeding of PEC with Antigen.** Thioglycollate-induced PEC were incubated in vitro for 45 min at  $37^\circ\text{C}$  in the presence of  $^{51}\text{Cr}$ -labeled TNP-substituted MRBC (TNP-MRBC) coated with either anti-MRBC or anti-TNP antibody, at a PEC to erythrocyte ratio of 1:10. After incubation, the mixture was washed, nonphagocytosed erythrocytes were lysed with 0.17 M  $\text{NH}_4\text{Cl}$  buffer, the remaining cells were washed several times in HBSS, counted in well-type gamma counter (Beckman Instruments, Munich, Federal Republic of Germany) and injected intravenously into recipients within 1 h.

**Sensitization to PCL or OX.** Mice were sensitized by applying 0.15 ml of a 7% solution of PCL or 3% OX in absolute ethanol to the skin of the clipped abdomen. 4 d later, unless otherwise stated, control ear thickness was measured with an engineer's micrometer (Moore and Wright, Sheffield, England), then both sides of both ears were painted with a 1% solution of homologous contact reagent dissolved in olive oil, and the increment of ear thickness was measured 24 h later. Results were expressed as the mean  $\pm$  SD in units of  $10^{-3}$  cm (9).

**Induction of CS Immunity or Unresponsiveness by Cell-bound Hapten.** CBA mice were injected with hapten-substituted syngeneic or allogeneic cells intravenously, subcutaneously, intraperitoneally, or into the footpad. Routinely (unless otherwise stated),  $3 \times 10^6$  epidermal cells,  $5 \times 10^6$  PEC,  $5 \times 10^7$  thymocytes (Thy), or  $10^9$  MRBC were injected. Some mice were treated with 50 mg/kg CY i.p. 24 h before injection of hapten-substituted cells.

Mice were tested for CS 7 d after the cell injection (unless otherwise stated), because it was found that they develop peak responses at this time. Mice found to be unresponsive were contact painted with corresponding or unrelated skin sensitizer and were tested 4 d later to ascertain if the unresponsiveness was the result of specific tolerance. Experiments in which ear swelling was measured at 12, 24, and 48 h showed that the peak response occurred 24 h after challenge in both skin-sensitized mice and those immunized with hapten-conjugated cells. Thus, only 24-h responses are shown in the Tables.

As a result of normal inter-experimental variability of ear swelling induced by contact painting in both sensitized and nonsensitized normal controls, the results were shown as the percentage of CS responses of contact-sensitized positive controls when data was summarized from several experiments. These were calculated for each individual experimental group according to the formula: the percentage of CS response =  $(A - C)/(B - C) \times 100$ ; where A refers to the 24-h increment in ear thickness in an experimental group that was immunized with conjugated cells; B refers to the 24-h increment in ear thickness in a positive control group which was immunized by contact skin painting; and C refers to the nonspecific 24-h increment in ear thickness of nonimmune controls.

### Results

*Induction of Immunity or Unresponsiveness by PEC-bound Hapten, According to the Route of Injection (Table I).* Hapten-conjugated PEC injected intravenously or intraperitoneally failed to induce CS, but when similar numbers of TNP-substituted (TNP-PEC) or OX-substituted PEC were injected subcutaneously (into the scapular region or into the footpads) mice had significant CS 1 wk later. The CS induced in this manner was hapten-specific (results not shown). Oil-induced syngeneic TNP-PEC were more immunogenic than thioglycollate-induced cells (group A vs. B) and allogeneic conjugated PEC did not induce CS (group C). TNP-substituted Thy (TNP-Thy) used in similar numbers as PEC (group D) induced insignificant CS, whereas higher numbers (group E) were moderately immunogenic. Thus, the induction of CS with hapten-conjugated PEC depends largely on the route of injection and the use of syngeneic cells.

*Induction of Specific Unresponsiveness by Cell-bound Hapten.* Experiments were conducted to see if the failure to induce CS by intravenous or intraperitoneal injection of hapten-conjugated cells was a result of the induction of tolerance. The results of such an experiment (Table II) show that mice injected intravenously with syngeneic or allogeneic PEC, Thy or erythrocytes labeled with TNP or OX could not be skin sensitized with appropriate sensitizer 7 d after injection of these cells. Unresponsiveness was specific because only responses to the homologous hapten were inhibited (Table II). However, suppression was less significant when OX was used to label the cells. Thus, neither the source of cells nor their genetic makeup was relevant to inducing specific unresponsiveness.

*Effect of CY on the Induction of CS (Table III).* Treatment of mice with CY (50 mg/kg) 24 h before the cell injection allowed animals injected intravenously with syngeneic, but not allogeneic TNP-PEC to develop significant CS reactions when tested 7 d later (group A). However, CY pretreatment failed to induce CS when either syngeneic or allogeneic TNP-Thy or TNP-MRBC were injected intravenously in similar numbers as macrophages (group B), although syngeneic cells used in higher

TABLE I  
*Induction of CS in CBA Mice by TNP-PEC\**

Group	Cell donor strain	Cells injected	Route of injection			
			Intravenous	Intraperitoneal	Subcutaneous	
					Footpad	Scapular
A	CBA	TNP-PEC (oil)	6.4 ± 3.8‡	10.7 ± 4.5	60.4 ± 10.8	70.5 ± 12.5
B	CBA	TNP-PEC (thioglycollate)	7.2 ± 2.7	12.3 ± 6.3	48.2 ± 3.9	55.4 ± 13.8
C	BALB/c	TNP-PEC (thioglycollate)	10.2 ± 5.3	8.8 ± 3.4	12.5 ± 1.4	10.8 ± 7.3
D	CBA	TNP-Thy ( $5 \times 10^6$ )	8.4 ± 2.0	11.2 ± 5.1	8.5 ± 3.7	10.4 ± 5.7
E	CBA	TNP-Thy ( $5 \times 10^7$ )	0.8 ± 1.0	8.4 ± 3.2	24.0 ± 5.8	28.5 ± 3.5
F	CBA	OX-PEC (oil)	3.0 ± 2.5	ND	66.7 ± 10.9	75.0 ± 17.8

ND, not done.

\* Mineral oil- or thioglycollate-induced hapten-substituted PEC ( $5 \times 10^6$ ) or thymocytes were injected by various routes and 7 d later, mice were tested for CS.

‡ Results are expressed as the percentage of CS response ± SD of contact skin-sensitized positive controls. The Table shows the combined results of four independent experiments in which each experimental group consisted of four to six animals.

TABLE II  
*Induction of Tolerance in CBA Mice by Injection of TNP- or OX-conjugated Cells\**

Group	Cell donor strain	Cells injected	CS 7 d after cells injected‡		CS 11 d after cells injected	
			Elicited by		Elicited by PCL 4 d after PCL painting‡	Elicited by OX 4 d after OX painting‡
A	CBA	TNP-MRBC	PCL	10.7 ± 5.6§	20.1 ± 5.3	98.4 ± 6.0
B	CBA	TNP-Thy	PCL	8.5 ± 6.4	12.0 ± 3.8	87.5 ± 7.6
C	CBA	TNP-PEC	PCL	6.6 ± 3.4	10.3 ± 4.7	92.4 ± 8.4
D	BALB/c	TNP-MRBC	PCL	12.3 ± 3.7	8.4 ± 3.3	110.1 ± 12.5
E	BALB/c	TNP-Thy	PCL	10.8 ± 5.0	11.1 ± 2.2	98.0 ± 4.2
F	BALB/c	TNP-PEC	PCL	4.9 ± 5.1	9.1 ± 2.0	102.3 ± 10.7
G	CBA	OX-Thy	OX	10.5 ± 3.5	92.8 ± 10.5	20.4 ± 8.3
H	CBA	OX-MRBC	OX	10.6 ± 2.9	106.0 ± 9.9	22.5 ± 7.1

\* CBA mice were injected intravenously with syngeneic (CBA) or allogeneic (BALB/c) cells ( $10^9$  TNP-MRBC,  $5 \times 10^7$  TNP-Thy, or  $5 \times 10^6$  TNP-PEC).

‡ Animals were tested for CS after 7 d or were sensitized by skin painting with corresponding or unrelated reactive hapten at day 7 and tested for CS 4 d later.

§ Results are expressed as the percentage of CS response ± SD of contact skin-sensitive positive controls. The Table shows the combined results of seven independent experiments in which each experimental group consisted of five or six animals.

TABLE III  
*Effect of CY on the Induction of CS in CBA Mice by TNP-conjugated Cells\**

Group	Cell donor strain	Cells injected	CS at 7 d	CS at 7 d in CY-injected mice
A	CBA	TNP-PEC ( $5 \times 10^6$ )	18.3 ± 4.5‡	60.8 ± 10.5
B	CBA	TNP-Thy ( $5 \times 10^6$ )	9.4 ± 6.0	18.3 ± 4.7
C	CBA	TNP-Thy ( $5 \times 10^7$ )	10.5 ± 3.5	31.2 ± 7.5
D	CBA	TNP-MRBC ( $10^6$ )	2.5 ± 2.0	10.5 ± 4.7
E	CBA	TNP-MRBC ( $10^9$ )	3.0 ± 2.5	20.0 ± 5.8
F	BALB/c	TNP-PEC ( $5 \times 10^6$ )	7.5 ± 3.3	15.0 ± 3.2
G	BALB/c	TNP-Thy ( $5 \times 10^7$ )	13.6 ± 7.4	16.7 ± 8.2
H	BALB/c	TNP-MRBC ( $10^6$ )	12.5 ± 3.9	12.5 ± 7.4

\* TNP-substituted syngeneic or allogeneic cells were injected intravenously into normal or CY-treated animals (50 mg/kg, 24 h before cell injection) and CS was tested 7 d later.

‡ Results are expressed as the percentage of CS responses ± SD of contact skin-sensitized positive controls. The Table shows the combined results from three independent experiments in which each experimental group consisted of five or six animals.

numbers induced some CS in CY-pretreated mice (groups C, D, and E). The CS was specific in that no increase in ear thickness occurred after challenge with OX, a non-cross-reacting hapten (Results not shown.). Thus, CY-sensitive regulatory cells were involved in the induction of tolerance by intravenous injection of hapten-conjugated syngeneic cells.

*Influence of Antibody on the Induction of CS by Cell-bound TNP (Table IV).* Injection of TNP-substituted cells intravenously does not cause the development of CS, but when the TNP-substituted cells were treated with antibody directed against TNP or against the cell to which the TNP was attached, significant CS could be induced. The reaction induced by antibody-treated TNP-substituted cells was specific in that no responses

TABLE IV  
*Influence of Antibody on the Induction of CS by TNP-conjugated Cells\**

Cell donor strain	Cells injected intravenously	Treatment of injected cells‡			
		No antibody	Anti-carrier antibody	Anti-TNP antibody	Anti-TNP (Fab') <sub>2</sub>
CBA	TNP-MRBC	3.5 ± 2.0§	48.5 ± 10.3	54.5 ± 7.4	5.0 ± 3.5
CBA	TNP-Thy	7.0 ± 3.5	57.2 ± 11.5	67.3 ± 10.8	12.3 ± 7.1
CBA	TNP-PEC	10.0 ± 7.1	ND	70.5 ± 12.6	11.4 ± 3.9
BALB/c	TNP-MRBC	5.3 ± 2.7	60.5 ± 10.4	52.6 ± 7.4	3.8 ± 3.2
BALB/c	TNP-Thy	5.8 ± 0.9	55.4 ± 7.5	58.2 ± 5.3	10.1 ± 5.4

ND, not done.

\* CBA mice were injected intravenously with TNP-substituted syngeneic or allogeneic cells ( $5 \times 10^6$  PEC,  $5 \times 10^7$  Thy, or  $10^9$  MRBC) and were tested for CS 7 d later.

‡ In some experiments, cells were treated before injection with anti-carrier or anti-TNP antibody, or with anti-TNP (Fab')<sub>2</sub> fragments.

§ Results are expressed as the percentage of CS responses ± SD of contact skin-sensitized positive controls. The Table shows the combined results of three independent experiments in which each experimental group consisted of five to seven animals.

TABLE V  
*Induction of CS by PEC That Had Phagocytosed Opsonized TNP-conjugated Erythrocytes*

In vitro cell combination before intravenous injection*	CS at 7 d
$5 \times 10^6$ PEC + anti-TNP antibody-coated TNP-MRBC ( $2.8 \times 10^6$ )	4.3 ± 0.89‡
$5 \times 10^6$ PEC + anti-MRBC antibody-coated MRBC ( $1.9 \times 10^6$ )	4.6 ± 0.56
$5 \times 10^6$ TNP-PEC + none§	1.5 ± 0.31
Positive control (7% PCL sensitization)	7.8 ± 1.23
Negative control (unsensitized)	1.4 ± 0.28

\* Thioglycollate-induced macrophages were incubated in vitro with <sup>51</sup>Cr-labeled TNP-MRBC (100 μCi/0.1 ml packed cells) coated with guinea pig anti-TNP or anti-MRBC antibody. PEC were freed of excess erythrocytes and injected intravenously into syngeneic recipients. The numbers of ingested erythrocytes, which are indicated within parenthesis, were estimated from PEC-associated radioactive counts.

‡ Results are expressed in units of  $10^{-3}$  cm ± SD. Each experimental group consisted of 6–10 animals.

§ PEC labeled directly with TNP were used as control cells.

to OX were noted (Results not shown.). Whereas, TNP-PEC injected subcutaneously, or TNP-substituted cells injected into CY-treated animals, only induced CS if syngeneic with the recipient, antibody treatment allowed syngeneic or allogeneic (BALB/c) cells to induce CS. Anti-TNP antibody raised in guinea pigs or mice (results not shown) facilitated the development of CS to the same degree. The integrity of the antibody molecule was essential because (Fab')<sub>2</sub> fragments were nonactive. Thus, the Fc fragment was critical, presumably by enabling macrophages in the immunized host to engulf the injected cells.

*Induction of CS by PEC that Phagocytosed TNP-substituted Cells (Table V).* When PEC were combined in vitro with TNP-MRBC which were complexed with antibody

TABLE VI  
*Induction of CS in CBA Mice by Hapten-conjugated Epidermal Cells*

Cell donor strain	Cells injected	Route of injection*	CS at 7 d‡
CBA	TNP-epidermal cells	Subcutaneous	6.4 ± 1.20
CBA	TNP-epidermal cells	Intraperitoneal	5.9 ± 0.36
CBA	TNP-epidermal cells	Intravenous	5.9 ± 0.83
CBA	Positive control (7% PCL sensitization)	—	8.3 ± 1.27
CBA	Negative control (unsensitized)	—	1.2 ± 0.34
BALB/c	TNP-epidermal cells	Intravenous	1.7 ± 0.30
BALB/c	Positive control (7% PCL sensitization)	—	6.7 ± 1.02
BALB/c	Negative control	—	1.3 ± 0.25
CBA	OX-epidermal cells	Intravenous	6.7 ± 1.24
CBA	Positive control (3% OX sensitization)	—	10.8 ± 2.05
CBA	Negative control (unsensitized)	—	2.0 ± 0.56
CBA	TNP-PEC ( $5 \times 10^6$ )	Intravenous	1.9 ± 0.56
CBA	TNP-epidermal cells ( $4 \times 10^6$ )	Intravenous	7.8 ± 1.04
CBA	TNP-PEC ( $5 \times 10^6$ ) + TNP-epidermal cells ( $4 \times 10^6$ )	Intravenous	6.1 ± 1.86
CBA	Positive control (7% PCL sensitization)	—	9.8 ± 1.84
CBA	Negative control (unsensitized)	—	2.9 ± 1.84

\*  $3 \times 10^6$  TNP- or OX-epidermal cells, syngeneic or allogeneic with CBA recipients were injected via different routes, and animals were tested 7 d later for CS.

‡ Results are expressed as increases in ear thickness in units of  $10^{-3}$  cm ± SD. Each experimental group consisted of six to eight animals.

directed against TNP or MRBC, and were then freed of erythrocytes and injected intravenously, they induced a significant level of CS. Thus, there is a substantial difference between PEC that were conjugated directly with TNP, and those that had ingested TNP antigen; only the latter were able to induce CS upon intravenous injection. These results also support the conclusion that antibody treatments (Table IV) allow induction of CS by promoting phagocytosis of the antigen.

*Induction of CS by Hapten Bound to Epidermal Cells (Table VI).* In contrast to the other cell types conjugated with hapten that, when injected intravenously, induced a state of unresponsiveness, TNP- or OX-conjugated epidermal cells injected intravenously induced CS. Conjugated epidermal cells had to be syngeneic with the recipient to induce CS. TNP-conjugated epidermal cells (TNP-epidermal cells) were also immunogenic when injected intraperitoneally or subcutaneously. Furthermore, TNP-epidermal cells induced CS even when injected intravenously with doses of TNP-PEC that induce tolerance. Thus, hapten-conjugated epidermal cells failed to suppress CS and uniformly induced CS, independent of the route of injection, and even when coadministered with doses of TNP-PEC that by themselves induced unresponsiveness.

Epidermal cells were fractionated by taking advantage of the fact that some of these cells (i.e., Langerhans cells [10]) have Fc receptors that promote adherence and rosette formation. Accordingly, epidermal cells were incubated with SRBC coated with mouse antibody and then were centrifuged in a Ficoll-Hypaque (Winthrop Laboratories, New York) gradient. The pellet contained 18% of the epidermal cells and was called Fc enriched, whereas the remaining cells at the interface were called

TABLE VII  
*Induction of CS by TNP-conjugated Epidermal Cells Fractionated According to Fc Receptors\**

Cells injected	Fraction	Number of cells $\times 10^6$	CS at 7 d‡	Number of mice
TNP-epidermal cells	Fc depleted	3	4.1 ± 1.2	4
TNP-epidermal cells	Fc enriched	1	6.7 ± 1.3	3
Positive control (7% PCL sensitization)	—	—	8.7 ± 1.63	5
Negative control (unsensitized)	—	—	2.0 ± 0.58	4

\* Epidermal cells were combined with antibody-coated erythrocytes and separated via density-gradient centrifugation into a pellet (Fc enriched) and an interface (Fc depleted) fraction. Subsequently, erythrocytes were lysed and remaining cells were conjugated with TNP and injected intravenously.

‡ Results are expressed as increase in ear thickness in units of  $10^{-3}$  cm ± SD.

TABLE VIII  
*Passive Transfer of CS Induced by Different Schedules of Sensitization\**

Induction of CS	Route of induction	CS at day 7	
		Actively sensitized donors	Passive transfer recipients
TNP-PEC	Subcutaneous	6.0 ± 1.20	4.1 ± 0.55
TNP-PEC	Intravenous	1.7 ± 0.56	1.6 ± 0.27
TNP-epidermal cells	Intravenous	5.9 ± 1.07	4.2 ± 0.34
Anti-TNP antibody-coated TNP-Thy	Intravenous	5.8 ± 0.86	4.4 ± 0.12
Positive control (7% PCL sensitization)	—	8.5 ± 1.36	6.1 ± 1.07
Negative control (unsensitized)	—	1.4 ± 0.33	1.3 ± 0.40

\* CBA mice were injected subcutaneously or intravenously with  $5 \times 10^6$  TNP-PEC, with  $3 \times 10^6$  TNP-epidermal cells, or intravenously with TNP-Thy complexed with anti-TNP antibody. Positive control animals were contact skin painted with 7% PCL. Lymph node and spleen cells were harvested 7 d after immunization (except when PCL was used, cells were collected at day 4) and injected intravenously into normal recipients ( $4 \times 10^7$  cells). Recipient animals were challenged immediately after transfer. Results are expressed in units  $10^{-3}$  cm ± SD. Each experimental group consisted of five or six animals.

Fc depleted. After erythrocyte lysis, both populations were conjugated with TNP and were used to induce CS by intravenous injection. The results of such an experiment (Table VII) show that Fc-enriched epidermal cells were more efficient at inducing CS than were Fc-depleted epidermal cells. Thus,  $1 \times 10^6$  Fc-depleted epidermal cells induced less than one-third of the CS responses induced by  $1 \times 10^6$  Fc-enriched epidermal cells. All the immunizing capacity of the epidermal cells could be obliterated by treatment with Ia antisera kindly donated by Dr. Donal Murphy of Yale University School of Medicine, New Haven, Conn. (Results not shown.).

*Passive Transfer of CS Induced by Different Schedules.* The experiments presented above showed that a similar CS could be induced by skin painting with PCL, by subcutaneous injection of TNP-PEC, by intravenous injection of TNP-Thy complexed with antibody, and by intravenous injection of TNP-conjugated epidermal cells. To confirm that in all cases we were dealing with a similar cell-mediated phenomenon, adoptive transfer experiments were performed. As shown in Table VIII, all of these modes of induction of CS provided sensitized cells which could transfer the ability to



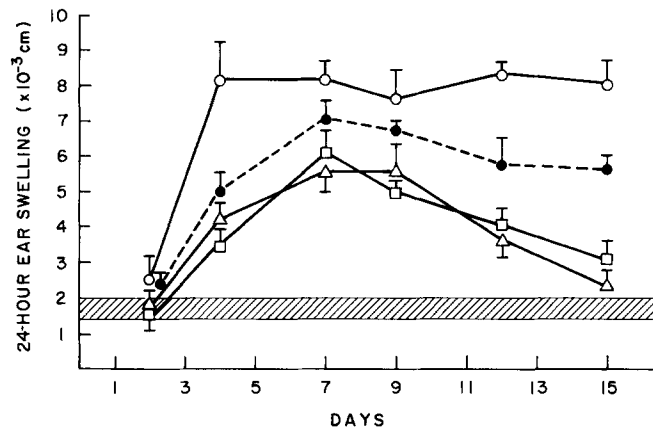


FIG. 1. On day 0, CBA mice were skin painted with 7% PCL (○) or injected with TNP-substituted cells ( $5 \times 10^6$  TNP-PEC subcutaneously [□],  $3 \times 10^6$  TNP-epidermal cells intravenously [●], or  $5 \times 10^7$  TNP-Thy complexes with anti-TNP antibody [△]). At different times thereafter, separate groups of animals were challenged with PCL and the ear skin swelling results were expressed in units of  $10^{-3}$  cm. Each experimental group consisted of five or six animals. The hatched area shows the range of nonspecific ear swelling in nonsensitized animals.

mount specific CS reactions to naive recipients, thus showing that a similar mechanism was involved in all cases.

*Time-Course of Elicited CS Induced by Different Modes of Immunization (Fig. 1).* CS reactions developed within 3–4 d in PCL contact skin-sensitized animals. By day 5 after PCL painting, maximal CS was elicited, and was equally elicitable over the next 10 d. In contrast, CS induced by TNP-PEC injected subcutaneously, or by intravenous TNP-Thy coated with anti-TNP antibody, was not maximal until 6–8 d, and then progressively declined; approaching responses in nonimmune animals by day 15. On the other hand, although CS induced by injection of TNP-epidermal cells also did not reach a maximum until day 6–8, they did not decline and therefore resembled in their lack of evanescence the CS induced by contact skin painting. Thus, contact painting or injection of TNP-epidermal cells induced CS that was long-lived, whereas TNP-PEC, or antibody-coated TNP-Thy, induced CS that was evanescent.

### Discussion

In this study, we have described three major outcomes of immunization with various forms of TNP: the induction of specific immunological unresponsiveness (tolerance) for CS, the induction of a short-lived or evanescent form of CS, or the induction of a long-lived form of CS.

The first outcome, the induction of specific immunological unresponsiveness, is probably the result of educating a net excess of suppressor cells that inhibits animals from making a CS immune response, and also makes them resistant to further immunization. As has been noted previously (3, 11), this occurs when initial immunization with TNP-conjugated cells occurs in the spleen via intravenous injection of hapten-conjugated cells. There is no requirement for major histocompatibility complex (MHC) identity between the haptenated cells and the host for this form of specific immunosuppression to occur, and presentation need not be on macrophages. However, we have found two ways to overcome this suppression: (a) allowing

phagocytosis of antigen to occur or (b) by using TNP-conjugated epidermal cells (see below).

The second outcome, induction of evanescent CS with an early peak followed by a rapid decline, occurs when the balance between suppression and helpers is skewed toward the latter situation by reduced activation of the suppressor circuit. There are three major ways to favor this skewing: (a) Pretreat the animals with a low dose of CY before their initial contact with antigen. CY, in the dose used, has been shown to inhibit cells in the suppressor T-cell circuit (11, 12). Our results in this case contrast with the findings of Miller et al. (13). Using a different hapten (dinitrophenyl), these authors found that pretreatment with CY at a much higher dose than we used (200 mg/kg) did not allow intravenously injected haptenated cells to induce immunity. The many differences between their experimental protocol and ours makes it difficult to identify the variable(s) that might account for the different results. However, because we (Table III), in agreement with Greene et al. (3), have noted that haptenated PEC are more immunogenic when suppression is avoided than are other types of haptenated cells, and because Miller et al. (13) used haptenated spleen cells in lieu of PEC, this might be the crucial difference. (b) Another way to reduce activation of the suppressor circuit is to pretreat the haptenated cells with passive antibody against either the hapten or the cell carrier. There are several possible explanations for this phenomenon. (i) Antibody may act by inducing increased phagocytosis of antigen because removal of the Fc piece from the antibody prevents it from fulfilling its role in inhibiting suppression, and feeding macrophages antigen before using them as immunogens also blocks their suppressor inducing qualities after intravenous injection. Once the antigen has undergone phagocytosis by macrophages, the antigen may be processed in a way which inhibits its ability to activate the suppressor circuit (perhaps as a result of antigen degradation as the work of Steinman and Cohn [14] has suggested) or favors induction of T cells which elicit DTH, as the work of Zembala et al. (15) has suggested. The conclusion that the host is processing antigen is supported by the observation that antibody coating of the TNP-conjugated cells is the only mode of sensitization in which TNP does not have to be syngeneic with the host to induce immunity. Presumably, after phagocytosis the antigen is presented to the immune system in association with the major histocompatibility determinants of the host's antigen-presenting cells. (ii) Antibody may alter the distribution of antigen within the lymphoid organs so that immunogenicity is enhanced and/or suppression reduced. An example of the latter is the retention of antigen-antibody complexes in the germinal center region of lymphoid organs. Chen et al. (16), using horseradish peroxidase (HRP) as a marker, have shown that small amounts of HRP-anti-HRP immune complexes formed in antigen excess escape phagocytosis and instead associate with the cell surface of nonphagocytic follicular dendritic cells. In this regard it is important to note that we have found that the most effective antigen presentation is performed by cells that are weakly phagocytic (Langerhans cells) or nonphagocytic (splenic dendritic cells, *vide infra*). (c) A third way of eliciting short-lived CS is by injecting the antigen-conjugated cells, that are predominantly macrophages, in such a way that primary immunization occurs in peripheral lymph nodes (3). This technique probably also works via bypassing suppression because it is well known that spleen-localizing T cells are much more likely to induce or act as suppressor cells than are cells that localize in the lymph node (17).

As noted by Greene et al. (3) there is a clear-cut hierarchy in the ability of different types of cells to immunize under those conditions which bypass suppression; PEC being significantly superior to Thy which are somewhat better than erythrocytes. It is unclear whether CY or antibody treatment blocks the suppression elicited by TNP-conjugated allogeneic cells, because MHC-incompatible cells fail to immunize. Thus, no reaction would be detected even if suppression had been alleviated.

The third outcome which may occur after immunization with TNP is the induction of a relatively long-lived form of immunity. This occurs after skin painting or more interestingly, when haptened epidermal cells are injected intravenously. Under these circumstances there is no need for blockage of the suppressor circuit for immunity to be seen. Whether the responses induced by this mechanism would be further augmented if suppression were to be reduced is now being tested.

The cell type(s) in the skin which are responsible for this potent form of antigen presentation are most likely Langerhans cells, because many of them appear to have Fc receptors (10). That the apparently Fc-negative skin cells can also induce this type of immunity, although less well than the Fc-positive fraction of cells, may be a result of contamination of the Fc-negative fraction, or skin cells themselves may have the ability to present hapten. This latter possibility is rendered unlikely because we have found that the epidermal cell type responsible for inducing long-lived CS bears surface antigens coded by the I region of the MHC. Tamaki et al. (18) and Rowden et al. (19) have noted that essentially all Ia-positive epidermal cells are Langerhans cells. We are presently attempting to see which I-subregion antigen(s) are involved, and preliminary results are in agreement with the finding of Tamaki et al. (18) and Rowden et al. (19) because both I-A and I-E/C antisera can eliminate the immunizing potential of the skin cells. Perhaps the most interesting question this work raises is: Why antigen presented on epidermal cells (most likely on Langerhans cells) induces such a different form of immunity than does antigen presented on peritoneal exudate macrophages? Several possible answers to this question come to mind:

(a) Within the PEC population there is a cell with an inducing quality similar to Langerhans cells but that their activity is not seen because of the fact that they are present only in very small numbers. The particular type of cells we have in mind would be a minimally phagocytic macrophage-like cell with cell surface antigens, coded for in the I region of the MHC (20, 21). Thus, we are trying to concentrate Ia-positive cells from various anatomical compartments to see if they have the same inducing qualities as do Langerhans cells.

One such cell is the splenic dendritic cell described by Steinman et al. (22). These cells are adherent and Ia positive, but lack Fc receptors and constitute <1% of splenic cells. Preliminary results indicate that enriched, TNP-conjugated splenic dendritic cells are able to function as potent antigen-presenting cells that can mimic Langerhans cells in their capacity to induce CS, even when injected intravenously, whereas enriched Fc-positive splenic macrophages that are conjugated with TNP mimic PEC macrophages in their inability to induce CS (W. Ptak and R. Steinman. Unpublished Results.).

(b) It is also possible that not all Ia-positive cells are the same, and that the Ia-positive cells in the skin will be different from Ia-positive antigen-presenting cells which are found in other anatomical compartments. If this proves to be the case, it would then be interesting and important to see if non-skin cells can be induced to

behave like skin cells by things other than the dermal-epidermal microenvironment. In particular, we have preliminary evidence that macrophages, stimulated by adjuvants that contain mycobacteria can acquire the immunity-inducing capacities of skin cells. The notion that this idea might be feasible, stems from the observations that (i) immunization with complete Freund's adjuvant leads to a long-lived immunity (2) and (ii) the studies of delayed type hypersensitivity by Mackaness et al. (2) and Lagrange and Mackaness (23) which indicate that the adjuvant effect of BCG leads to the induction of a set of cells which produce additive effects when mixed with cells immunized to express evanescent DTH. Thus, the questions are: Are all Ia-positive antigen-presenting cells the same? If not, what are the differences, and what experimental techniques are available to help us understand the mechanism by which some of these cells become superior inducers of a CS response?

It is also important to determine why some modes of antigen presentation can induce immunity without the need for inhibition or bypassing of the suppressor circuit. Because TNP-epidermal cells are able to induce CS when injected intravenously mixed with TNP-PEC, this implies that Langerhans cells can effectively present antigen in the presence of suppression, whereas immunity-inducing, antigen-presenting cells in PEC cannot. This might suggest that Langerhans cells are able to activate effector T cells that are relatively resistant to suppression, or alternatively, that the specialness of these antigen-presenting cells is their inability to interact with the cells of the suppressor circuit.

In any case, it is quite interesting to note that CS comes in the same two forms as other forms of DTH and that the type of immunity which will evolve depends upon a complex interplay between three major classes of cell types: the antigen-presenting cells, the DTH effector cells, and the cells in the regulatory suppressor cell circuit (24). The three major factors that have been identified so far which can significantly alter the normal interplay are: (a) the anatomical site where the initial immunizing procedure occurs; (b) the presence or absence of a subset of cells in the suppressor circuit that are highly sensitive to CY; and (c) the nature and origin of the cell which presents the antigen.

### Summary

Three outcomes pertinent to contact sensitivity (CS) follow immunization with various forms of trinitrophenylated (TNP) substrates: (a) specific immunological unresponsiveness for CS is induced when immunization favors activation of splenic suppressor cells. This state is achieved by intravenous injection of trinitrophenyl-conjugated to various types of cells, such as peritoneal exudate cells (PEC). (b) A short-lived or evanescent form of CS is induced when immunization reduces activation of the suppressor circuit. This can be achieved by subcutaneous immunization with trinitrophenyl conjugated to syngeneic PEC, by pretreatment with cyclophosphamide to diminish suppression before intravenous immunization, or by altering the mode of antigen presentation by using TNP-substrate that has undergone phagocytosis. (c) A long-lived form of CS is induced when trinitrophenyl is presented to the immune system on skin cells either by contact skin painting with reactive trinitrophenyl, or by subcutaneous, or even intravenous injection of trinitrophenyl-conjugated epidermal cells. In fact, trinitrophenyl-conjugated epidermal cells induced CS even when the suppressor circuit was activated by intravenous coadministration of TNP-PEC. This

implies that antigen presentation on epidermal cells induces sensitized cells that are relatively resistant to suppression. The cell type(s) in the skin that are primarily responsible for this potent form of antigen presentation are most likely Langerhans cells, because they can be concentrated by virtue of their Fc receptors and they are Ia positive. Thus, both the anatomical site where antigen is first encountered by the immune apparatus, as well as the nature of the cells which present the antigen, determine whether a CS response will ensue, as well as whether it will be evanescent or long-lasting.

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