trol animals showed a considerably more effective antibody response to horse serum than untreated controls. Bacterial infection, usually in the form of abscesses in the region of the ablated thymus or broncho-pulmonary inflammation, was noted in all wasted animals. A predominantly plasmacytic inflammatory reaction and elevation of serum gamma globulin level were observed in most wasted neonatally thymectomized rats in spite of their lymphopenia and splenic atrophy. The findings suggest that bacterial infections, presumably acquired early in life, contribute to the subsequent development of immunologic deficiency and wasting in neonatally thymectomized rats.

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In vitro Cytotoxicity in Delayed-Type Hypersensitivity and Immunologic Tolerance.* (29384)

D. M. LUBAROFF† AND R. E. RITTS‡

Department of Microbiology, Georgetown University, School of Medicine, Washington, D.C.

Since the early observations of Holst(1) and the classic work of Rich and Lewis(2,3) with tuberculin, an impressive body of evidence has accumulated in support of the phenomenon of *in vitro* toxicity of an antigen for explanted cells from a donor exhibiting delayed-type hypersensitivity. These so-called cytolytic studies have been extended to a number of microbial delayed-type reactivities including streptococcal M substance (4), histoplasmin(5), mumps antigen(6), and brucellergin(7). The only investigation using a haptenic system in which delayed skin reactivity may be selectively engendered was that of Everett *et al* who failed to note any toxic effect on epithelial cells with 2,4 dinitrochlorobenzene or Rhus oleoresin(8). There have been other such negative results with microbial antigens where cells of epithelial origin were used(9,10). All of these cytolytic or cytotoxic studies have served as the sole *in vitro* measure of delayed-type reactivity.

The experiments to be described were designed to observe whether leukocytes from

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A portion of this study was submitted in partial fulfillment of requirements for the M. S. degree, Georgetown University. Present address: Dept. of Microbiology, Yale Univ., New Haven, Conn.

‡ Present address: AMA Inst. for Biomedical Research, Chicago, Ill.

animals with delayed-type contact hypersensitivity would be affected by the hapten engendering this reactivity and further, to study the effects of a hapten on cells from animals that had been rendered specifically tolerant. For both of these purposes picryl chloride ("PCl"; 2,4,6-trinitrochlorobenzene) served as the hapten.

Materials and methods. Animals. Randomly bred, albino guinea pigs§ weighing 300-400 *g* were housed 3-4 to a cage, fed oats, guinea pig chow, chopped cabbage and kale, bedded on timothy hay and maintained at 72°F. Where indicated, all animals were sensitized to PCl by a multiple intradermal injection scheme(11). Immunologic unresponsiveness or tolerance to PCl was induced after the method of Chase by daily oral administration of 0.3 ml of a 1% solution of PCl in corn oil by infant plastic feeding tube(12,13).

Skin tests were made 2 weeks after the last sensitizing dose; hypersensitive, fed, and normal animals underwent this procedure. Skin test doses consisted of a standardized drop of 1%, 0.3%, and 0.1% PCl in olive oil plus a fourth site for the olive oil vehicle (toxicity control) each on a separately clipped and shaved skin site on the flanks. After 24 hours the hair bridge separating the 2 sites on each side was removed, the areas washed with warm soapy water and the reactions scored at 24 and 48 hours on the basis of induration and color(11). Normal control animals were either injected with saline or fed with corn oil alone. All control animals were skin tested.

Any guinea pig selected to be a cell donor was either a ++++ or a +++± reactor from the sensitized group or a negative or faint trace reactor in the unresponsive group. All control animals were negative to PCl. Thus in the experiments to be described the cells were from donors which exhibited marked delayed-type skin response to PCl (sensitive group) or showed no reactivity to PCl (fed-tolerant and normal groups). No animal was used as a cell donor until at least 2 weeks had elapsed since the last skin test. Frequently this was a matter of one or two months. Once established, both unresponsiveness and sensitivity are durable for many months in spite of the absence of the antigen and no antipicryl antibody is produced (14).

Cell suspensions: All glassware used in handling of cells was siliconed‖ and sterilized in a steam autoclave. Normal, sensitive and tolerant animals were killed by bleeding from the heart with a 20-gauge needle and syringe. The abdominal surface of the guinea pig was clipped, washed with soap and water and sponged with alcohol. The axillary, mesenteric and inguinal lymph nodes were dissected out under sterile conditions, trimmed free of fat and placed in a petri dish containing 20 ml of Hanks'-Basal Medium Eagle's solution (H-BME).¶ After rupturing the nodes and teasing out the cells, the leukocyte-rich fluid was centrifuged at 2000 rpm for 15 min at 4°C in a conical bottom centrifuge tube. Decanting the supernatant, the cells were resuspended and washed twice in 10 cc of the nutrient solution. The final cell button was resuspended in 2 ml of H-BME solution and after counting the cells, 20 tubes for each animal were prepared to contain 10^6 leukocytes (92% lymphocytes) in 5 cc of H-BME supplemented with 5% normal guinea pig serum previously heated to 56°C for 35-45 min. Thrice-recrystallized PCl in saline(11) was added to the cell suspensions in 0.2 cc amounts containing 25, 10, 5, 2, 1, or 0.3 μg. The 3 higher concentrations proved to be non-specifically toxic to normal cells although there were clear differences between them and hypersensitive cells. The lowest amount was found to cause no discernible effect on cells secured from normal or sensitive donors, when studied under the conditions noted.

Each of the 16 × 150 mm screw cap tubes was placed in a roller drum (0.25 rpm) at 37°C. At zero time (PCl addition), at 15 minutes, and at hourly intervals for 4 hours, 4 tubes per cell donor animal were removed and the cell suspension transferred to 15 cc conical bottom tubes. After centrifugation

§ Obtained from Doval Farms, College Park, Md., and Evans Rabbitry, Washington, D.C.

‖ Beckman Desicote.

¶ Microbiological Associates, Bethesda, Md.

TABLE I. Effect of PCl on Lymphocytes from Normal and Hypersensitive Guinea Pigs.

Length of incubation (hr)	Avg Δ_{AC} 12 normal guinea pigs*	Avg Δ_{AC} 11 hypersensitive guinea pigs†	Probability
$-\frac{1}{2}$	0	0	—
0‡	0	0	—
$+\frac{1}{3}$	−1.54	3.36	1–2%
1	2.14	10.05	<1.0%
2	4.55	18.80	<1.0%
3	7.80	32.40	< .1%
4	12.10	36.10	< .1%

Each value represents quadruplicate samples for each cell donor at each time period, yielding a total of

* 48 samples and

† 44 samples respectively.

‡ 2 μg PCl/saline added to cells.

at 2000 rpm for 10 minutes at 4-5°C the cells were resuspended in 0.5 ml H-BME for total cell counts and viability determinations using trypan blue(15). Anti-PCl antibody was sought for in cell donor's serum by precipitin and Ouchterlony techniques(16) using PCl-BGG conjugate(11). No antibody to PCl could be detected.

Results. Total cell counts were unreliable as an index of the cytotoxic phenomenon, since under phase microscopy there were intact but presumably non-viable cells after contact with PCl. Accordingly, the difference of per cent viability of "sensitive" or "tolerant cells" *vs* "normal cells" in the presence of the PCl was determined in quadruplicate for each animal at each of the indicated times. The results are expressed as the delta values (Δ_{AC}) for each time period. This was calculated by subtracting the average per cent viability of cells maintained in the presence of antigen (A) from similar viability counts of replicate cell suspensions in the absence of antigen (C) for each animal.

% CELLS VIABLE = [(NO. CELLS EXCLUDING TRYPAN BLUE)/(TOTAL CELLS)] × 100

DELTA (Δ) = % CELLS VIABLE *WITHOUT* PCl MINUS % CELLS VIABLE *WITH* PCl

DELTA (Δ) = 90%-50%

DELTA (Δ) = *40*

Repeat viability determinations were within 7%. The data presented are the average figures for 12 normal, 11 hypersensitive and 8 tolerant or unresponsive animals. Additional data on specificity are from 4 normal and 4 hypersensitive animals.

The data (Table I) indicated that while PCl did alter the cell population of both normal and hypersensitive cells there was a rather prompt effect on sensitive cells resulting in about a 10% death rate at one hour. However at 2 hours ($p<0.01$) and 3 and 4 hours ($p<0.001$) a significant death rate had occurred in the sensitive cell groups, suggesting that prolonged exposure might be required or, more probably, that of those cells that were truly competent in the total cell population of each animal, there were varying degrees of "susceptibility" to the PCl. Control suspensions of sensitive leukocytes in the absence of antigen behaved as normal cells during the time they were observed.

The tolerant cells (derived from those prefed guinea pigs known to be specifically unresponsive to PCl after a course of sensitization) were then subjected to the identical test system. The data (Table II) indicated that these cells were not affected by the PCl ($p<0.001$).

If these observations were to have meaning, a heterologous but similar hapten should exert no effect on the cytotoxic system. To study this possibility 2,4 dinitrochlorobenzene (DNCB) was chosen as a suitable substance to test. Twice recrystallized DNCB in the same amount as in the PCl experiments was added since the ratio of haptenic molecules/cell numbers would be about the same. The results (Table III) showed that

TABLE II. Effect of PCl on Lymphocytes from "Unresponsive" and Hypersensitive Guinea Pigs.

Length of incubation (hr)	Avg Δ_{AC} unresponsive guinea pigs	Avg Δ_{AC} hypersensitive guinea pigs	Probability
$-\frac{1}{2}$	0	0	—
0*	0	0	—
$+\frac{1}{3}$	.10	3.36	5–10%
1	.02	10.05	<.1%
2	3.64	18.80	<.1%
3	3.92	32.40	<.1%
4	12.05	36.10	<.1%

* 2 μg PCl/saline added to cells.

TABLE III. Effect of DNCB and PCl on Cells of PCl-Sensitized Guinea Pigs.

Length of incubation (hr)	$Delta_{AC}$ with DNCB	$Delta_{AC}$ with PCl	Probability
−½	0	0	—
0	0*	0†	—
+⅓	−1.5	3.36	5–10%
1	−1.7	10.05	<.1%
2	− .5	18.80	<.1%
3	2.0	32.40	<.1%
4	4.6	36.10	<.1%

* 2 μg DNCB/saline added to cells.
† 2 μg PCl/saline added to cells.

cells derived from markedly PCl sensitive guinea pigs were not injured by DNCB and were essentially the same as normal cells ($p<0.001$).

Discussion. These experiments have indicated that cells secured from guinea pigs exhibiting marked delayed-type skin reactivity to a hapten, PCl, are killed but not necessarily lysed at a faster rate and in greater numbers than cells from a normal animal when exposed to PCl in an *in vitro* suspension system. Lymphoid cells of guinea pigs rendered specifically unresponsive to PCl were not affected by this antigen under identical *in vitro* conditions.

While it was appreciated that only some proportion of cells from the sensitive animals could be expected to be competent with respect to PCl sensitivity it was expected that any effect PCl would have in the *in vitro* system would probably be prompt and that no continuing effect would occur. However, the results indicated that while there was a prompt killing of some cells it is quantitatively small. The maximum effect appears to occur after 2 hours. Several more experiments not reported here were carried on to 8 hours but there was no further significant effect.

The cytotoxic effect observed with this hapten occurred in a rather restricted range. Two and one-half times the amount reported here killed normal cells, but significantly less than "sensitive" cells, while 5 or 12½ times caused some lysis and little discernible difference between cells from normal and hypersensitive donors. These data are interpreted as the manifestation of inherent toxicity of PCl.

Cell suspensions were chosen for this study rather than the explant technique because it is not usually possible thoroughly to wash cells in lymphoid tissue fragments or nodes and a number of materials, especially antibody, if present, are free to react. Further, the latter method measures cell migration and it is not certain if the cell types, principally fibroblasts and macrophages, that are being measured are truly involved in the *in vivo* circumstances.

There have been some conflicting views on the necessity for complement in this or related phenomenon. Miller and Favour and their colleagues have suggested that complement is required(17) and that a plasma factor mediates cytolysis(18,19). However, Waksman(20) and Wesslen(21) have not found complement or a plasma mediator to be involved. In these experiments all serum added to the H-BME solution was heated and the cells thoroughly washed. Consequently it was unlikely that complement could be implicated. Similarly no antipicryl antibodies were found to be participating in the cytotoxicity.

In pilot studies using H^3PCl it was determined that there was a small (not greater than 10% of the total) amount of PCl that became conjugated to the guinea pig serum in the maintenance solution. However it is not known whether the free or the conjugated PCl was the effective antigen. The reported cytotoxicity to PCl appears to be a remarkably specific one since DNCB caused no effect on the cells from hypersensitive donors.

Lymphoid cells from animals rendered specifically tolerant to PCl were not affected *in vitro* by PCl. These data appear to corroborate the earlier suggestion that the immunologic defect in unresponsiveness is expressed as a failure of cellular participation (14,22). This would be consistent with the findings in immunologic paralysis to pneumococcal polysaccharide(23,24) and tolerance resulting from protein overloading(25).

These results appear to be consonant with the observations of Kirchheimer and Weiser who were unable to demonstrate cytolysis to tuberculin when sensitive cell donor animals

had been desensitized(26). While their results are similar to the data reported herein utilizing cells from tolerant animals, the mechanisms by which cells are rendered inert in the two situations make further speculation unwarranted.

Summary. Picryl chloride was found to exert a lethal effect *in vitro* on leukocytes, principally lymphocytes, from guinea pigs exhibiting delayed-type skin reactivity to this hapten in the absence of antibody and complement. This reaction was found to be immunologically specific. PCl caused no discernible effect on leukocytes from guinea pigs rendered specifically tolerant to this substance.

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Trachoma Viruses Isolated in the United States. VIII. Separation of TRIC Viruses from Related Agents by Immunofluorescence.* (29385)

LAVELLE HANNA AND HANS BERNKOPF (Introduced by Ernest Jawetz)
Department of Microbiology, University of California, San Francisco Medical Center, San Francisco

For many years the similarity of trachoma-inclusion conjunctivitis (TRIC) viruses to agents of the psittacosis-LGV group has been evident on the basis of size, morphology, developmental and chemical characteristics and the possession of a common "group" antigen. The behavior of all these organisms in the commonly employed serologic tests (complement-fixation, neutralization) has been so uniform that the antigenic expression of species-specificity has been questioned. Such species-specific antigens were first demonstrated in the cell walls of meningopneumonitis (MP) organisms by Jenkin(1), but no similar specific antigens have yet been obtained from TRIC agents. The serologic results of Woolridge *et al*(2), which appeared

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