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Immunology

Transfer of an antigen-specific immediate hypersensitivity-like reaction with an antigen-binding factor produced by T cells

(T lymphocyte/immediate hypersensitivity/delayed-type hypersensitivity)

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ABSTRACT **The fact that T cell-dependent activation of mast cells occurs in delayed-type hypersensitivity led us to investigate whether a T cell product could mimic some of the functions of IgE. We report that 24- or 48-hr cultures of T cells from mice immunized optimally for delayed-type hypersensitivity resulted in release of an antigen-binding factor that transferred the ability to elicit an antigen-specific immediate hypersensitivity-like skin reaction in normal recipients. The responsible factor was concentrated and purified by affinity chromatography on antigen columns and was distinguished from immunoglobulin by several criteria: (*i*) it was released by purified T cells (anti-immunoglobulin plate depletion of B cells); (*ii*) it expressed no known antigenic markers of immunoglobulins (enzyme-linked immunosorbant direct binding assay); (*iii*) it had a molecular weight of 70,000 or less (sucrose gradient ultracentrifugation); and (*iv*) it had serological markers associated with antigen-specific T cell factors from other experimental systems. We suggest that, at sites of delayed-type hypersensitivity, antigen-reactive T cells may release antigen-specific factors that lead to mast cell activation and release of vasoactive amines, which is required for elicitation for these responses.**

The immune response that takes place in the skin of antigen-challenged sensitized subjects has been divided into major classes by virtue of the time it takes for a detectable reaction to appear after the antigenic challenge. On one end of the scale are immediate hypersensitivity reactions, which have been shown to be due to the rapid release of vasoactive amines and other mediators from degranulated mast cells that have been passively sensitized with specific antibody of the IgE isotype (1–3). On the other end of the spectrum are cell-mediated hypersensitivity reactions, in which antigen-specific immune Lyl T cells recruit other inflammatory cells to the site of the antigen (4). In this case the series of events that take place before a visible or measurable reaction appears usually requires about 24 hr. Thus these latter reactions have been called delayed-type hypersensitivity. Conventional immunological dogma separates immediate and delayed-type hypersensitivity reactions from one another and implies that they are unrelated.

However, some recent experiments suggest that this dogma might need modification. In particular, it has been shown that inhibition of the release of vasoactive amines from mast cells can inhibit both immediate and delayed-type hypersensitivity (5) and that T-cell dependent degranulation of mast cells occurs in delayed-type hypersensitivity (6). These findings in murine models have been interpreted to mean that both the protein-rich fluids that exude from local blood vessels in immediate hypersensitivity and the inflammatory cells that migrate from the blood to the site of delayed-type hypersensitivity require that vasoactive amines produce gaps between endothelial cells so that the relevant molecules, cells, or both responsible for the two reactions can enter the tissue spaces. Because mast cell release of vasoactive amines is required in both reactions, and because delayed-type hypersensitivity is due to T cells, we reasoned that a T cell-released product should mimic, at least in part, some of the vasoactive amine-releasing functions of IgE.

In this report we show that supernatants of cultured T cells from immunized mice contain an antigen-binding material that can be distinguished from immunoglobulin by virtue of the facts that: (*i*) it expresses no known antigenic markers that have been associated with immunoglobulin; (*ii*) it is considerably smaller than immunoglobulin; and (*iii*) it bears antigenic markers associated with T cell factors. This T cell-derived antigen-binding factor can passively sensitize mice after intravenous injection in a way that will allow elicitation of a measurable skin reaction within 30 min after challenge with the specific antigen.

MATERIALS AND METHODS

Mice. Male CBA/J mice 5–6 weeks of age were obtained from Jackson Laboratories and were rested at least 1 week in a Bioclean facility before use.

Reagents. Picryl chloride (PCL) (Chemotronix, Swannanoa, NC) was recrystallized three times from methanol/H_2O before use and was protected from light during storage. Bovine gamma globulin (BGG), and ε-amino caproic acid (EACA) were purchased from Sigma. Rabbit anti-mouse light and heavy chain isotype reagents were purchased from Litton Bionetics (Rockville, MD) and Gateway Immunoserum (St. Louis, MO). Their specificity was tested by Ouchterlony double diffusion analysis against myeloma proteins (Litton Bionetics). Hapten affinity-purified murine monoclonal anti-2,4-dinitrophenyl IgE and monospecific rabbit anti-IgE serum were gifts from David H. Katz and F.-T. Liu. Goat anti-rabbit immunoglobulin (Ig) for enzyme-linked immunosorbant assay (ELISA) testing was obtained from Cappel Laboratories, Cochranville, PA as a lyophilized serum.

General Methods. Affinity chromatography was performed with Sepharose 4B (Pharmacia) as the support matrix. Proteins were covalently attached to Sepharose by the CNBr technique (7). Goat anti-mouse Ig was affinity purified by absorption to and elution from mouse Ig-Sepharose 4B columns and was used for *in vitro* depletion of B cells from mixed cell populations and as a reagent for ELISA testing. Mouse Ig used for this purification was prepared by salting out pooled normal mouse serum with 18% Na_2SO_4 (wt/vol). 2,4,6-Trinitrophenyl (TNP) was co-

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Abbreviations: BGG, bovine gamma globulin; EACA, ε-amino caproic acid; ELISA, enzyme-linked immunosorbant assay; HRP, horseradish peroxidase; i.v., intravenous(ly); PCL, picryl chloride; PCL-F, PCL factor; P_i/NaCl, 0.01 M sodium phosphate-buffered saline (pH 7.4); TNP, 2,4,6-trinitrophenyl; TSF, T-cell suppressor factor.

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valently attached to BGG-Sepharose by reaction with trinitrobenzene sulfonic acid in 0.2 M Na_2CO_3 at pH 11. A ratio of 10 mg of trinitrobenzene sulfonic acid for each 10 mg of BGG was used. TNP-EACA was prepared by reaction of trinitrobenzene sulfonic acid with EACA in 0.2 M Na_2CO_3 at pH 11. TNP-EACA was extracted from the acidified (pH $\leq$ 2.5) reaction mixture with ethyl acetate and crystallized from methyl alcohol/H_2O.

Contact Sensitization and Production of Antigen-Binding Factor from PCL-Sensitized Cells. Donor CBA/J male mice (8–10 weeks old) were sensitized by topical application of 0.15 ml of 5% PCL in absolute ethanol and acetone, 3:1 (vol/vol), to the shaved abdomen, chest, and four feet. Four days later spleen and peripheral lymph node cells from these mice (approximately 1–2 $\times$ 10^8 cells per mouse) were cultured in vertical 250-ml flasks (70–150 ml per flask) at 1.5 $\times$ 10^7 cells per ml in serum-free RPMI 1640 medium (GIBCO), supplemented with mycostatin, gentamicin, and streptomycin, for 24 or 48 hr at 37°C under a 5% CO_2 atmosphere. The culture fluid supernatant was then separated from the cells by centrifugation for 7 min at 25°C at 2,500 rpm in an HG 4L rotor (Sorvall) and antigen-binding material (hereafter called PCL factor, PCL-F) was purified by addition of the culture fluids (100–200 ml) to an affinity matrix composed of TNP-BGG-linked Sepharose beads followed by overnight incubation of the mixture at 4°C with gentle stirring. The mixtures were then poured into columns over a bed of Sephadex G-25 and washed with 5 column volumes of sodium phosphate-buffered saline (P_i/NaCl). The columns were then eluted at 4°C with 0.05 M TNP-EACA. In some experiments 5 M guanidine was used for elution. The eluate in a volume of about 5–10% of the original supernatant was then exhaustively dialyzed at 4°C vs P_i/NaCl. PCL-F was dialyzed into 0.15 M NaCl for intravenous (i.v.) transfer. The protein content (8) of purified PCL-F ranged between 0.1 and 0.3 mg/ml for the 12 preparations employed in this study. In a typical experiment supernatant obtained from lymphoid cells of 50–100 mice resulted in a 2-mg yield of hapten affinity-purified PCL-F protein.

Bioassay of PCL-F. Unless otherwise stated, 75 μg of PCL-F in 0.5 ml of 0.15 M NaCl was injected i.v. via the retroorbital plexus into lightly ether-anesthetized mice and both sides of their ears were challenged immediately thereafter by topical application of a drop (27 gauge needle) of 0.8% PCL in olive oil. In one experiment 0.8% PCL was applied to one ear and 0.8% oxazolone (Gallard Schlesinger, Carle Place, NY) in olive oil was applied to the other. Duplicate measurement of ear thickness was made with an engineer's micrometer (Mitutoyo, Tokyo, Japan) prior to challenge and at various times from 0.5 to 24 hr later. The increment in ear thickness was expressed as the mean $\pm$ SEM in units of 10 μm (9). In each experiment the ears of a separate group of noninjected controls were challenged and measured similarly to those of mice that received PCL-F.

Immunoassay by ELISA. Horseradish peroxidase (HRP) obtained from Sigma was coupled to goat anti-rabbit Ig by using glutaraldehyde (10). Rabbit Ig (100 ng) was coated onto the wells of microtiter trays (Linbro Plastics). Dilutions of goat anti-rabbit-HRP conjugate (0.1 ml) in 0.05% Triton X-100/P_i/NaCl were incubated with coated wells for 3 hrs at 20°C. A dilution of conjugate was chosen that produced an A_{492} value of 1.0 when developed after interaction with substrate. Conjugates were stored in 50% (vol/vol) glycerol in 20-μl aliquots at −70°C.

To test PCL-F for the presence of Ig determinants, a direct binding assay was used: it consisted of coating each of the 12 different preparations of purified PCL-F used in this study, or mouse immunoglobulin standards, onto the wells of flat-bottom polystyrene microtrays (Linbro Plastics) at a concentration of 100 ng per well in 0.01 M sodium borate buffer (pH 8.6). The coating was allowed to proceed for 18 hr at 4°C. Trays were then washed with distilled water, dried at 37°C, and stored at 4°C under reduced pressure and over desiccant until used. Various test antisera (anti-μ, anti-κ, anti-γ, anti-ε, etc.) and control pooled nonimmune rabbit sera were diluted into 0.05% Triton X-100/P_i/NaCl in 2-fold steps starting at 1:20. Duplicate aliquots of diluted test antisera (0.1 ml) were incubated with PCL-F or Ig-coated wells for 18 hr at 4°C. Trays were then washed four times with 0.05% Triton X-100/P_i/NaCl and blotted dry, and 0.1 ml of HRP-conjugated goat anti-rabbit Ig was added at the dilution determined above. Incubation with HRP-goat anti-rabbit Ig was for 3 hr at 25°C. The trays were again washed four times with 0.05% Triton X-100/P_i/NaCl and 0.2 ml of substrate was added. The substrate used for these studies was *o*-phenylenediamine (Sigma) and was prepared by the method of Voller *et al.* (11), in 0.1 M phosphate-citrate buffer (pH 5.0). Color development proceeded for 20 min, at which time the reaction was terminated by the addition of 0.05 ml of 6 M HCl to each well. Color was determined quantitatively on a Titertek Multiscan (Flow Laboratories, McLean, VA) at 492 nm. It was estimated that this assay could detect at least 5–10 ng of any Ig antigen per ml of test sample.

Production of Rabbit Anti-Factor Antisera. Antisera were produced in collaboration with Robert Cone. Rabbits were injected with 200 μg of PCL-F or T cell-derived suppressor factor (TSF) (12) emulsified in complete Freund's adjuvant and were boosted with PCL-F or TSF and poly(A)·poly(U) (Boehringer Mannheim) (13). Antisera obtained were routinely screened for anti-mouse Ig heavy and light chain isotypes and anti-hapten activity by ELISA. Anti-PCL-F sera used were negative for binding to Ig determinants but always contained anti-TNP activity. This anti-TNP activity was absorbed out by passage over TNP-BGG-Sepharose absorbents, and this absorbtion was confirmed by ELISA employing TNP-BGG-coated plates.

B Cell Depletion of Mixed Lymphoid Cells. A modification (13) of the technique of Wysocki and Sato (14) that employed goat anti-mouse Ig-coated plates was used.

Sucrose Density Gradient Analysis. Discontinuous sucrose gradients were used to fractionate PCL-F by size. The gradients consisted of 2.5 ml of 5%, 10%, 15%, 20%, and 25% sucrose in distilled water. Samples (0.5 ml) were layered on each gradient, and then centrifuged at 35,000 rpm in an SW 41 rotor (Beckman) for 18 hr at 10°C. Gradients were fractionated by pumping out the contents of gradient tubes, using an LKB Varioperpex 2120 pump. Molecular weights were determined by standards: MOPC 104E (IgM, 900,000); TNP-BGG (150,000–200,000); bovine serum albumin (70,000); and ovalbumin (45,000).

RESULTS

PCL-Immune Cells Release an Antigen-Specific Factor (PCL-F) that Can Transfer an "Immediate Hypersensitivity"-Like Response. Antigen (TNP)-binding material was released into culture supernatants by lymphoid cells of mice that were contact sensitized with PCL. After purification and concentration by hapten affinity chromatography, i.v. transfer of this material (hereafter called PCL-F) to normal nonimmune mice resulted in the ability to elicit an immediate hypersensitivity-like contact ear swelling response with PCL. This response was not elicited by PCL challenge in noninjected controls (Fig. 1). The ear swelling response in PCL-F recipients was detected as early as 30 min after challenge, was maximal at 1–4 hr, and then declined to levels comparable to noninjected controls by 18 hr after challenge (Fig. 1). The dose of PCL-F used for transfer was 75 μg per mouse in the 11 experiments shown in Fig. 1. Two dose–response experiments were performed (data not shown). In one experiment recipients manifested an immediate ear

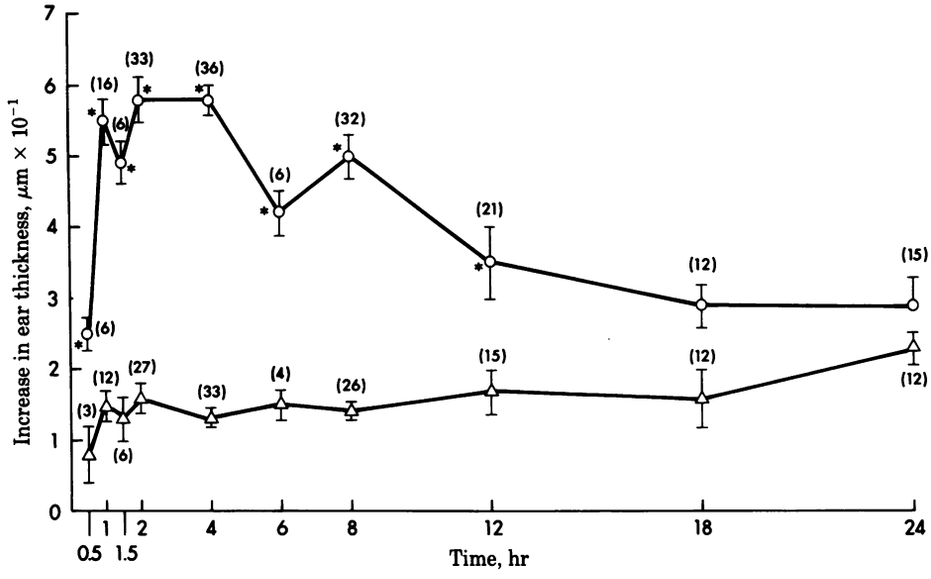

FIG. 1. Time course of contact ear swelling response in mice receiving PCL-F. Separate groups of male CBA/J mice were injected i.v. with 75 μg of hapten affinity-purified PCL-F (○) or received nothing (△). Immediately thereafter mice were challenged on the ears with 0.8% PCL in olive oil. The figure shows the time course of the resulting ear swelling response in 11 independent experiments that employed 9 different PCL-F preparations in 3–6 mice per group. The total number of mice used to calculate the mean ± SEM is given in parentheses at each time point. The ear swelling was measured with a micrometer.
* Statistically significant difference ($P < 0.005$) between PCL-F injected mice and noninjected controls.

swelling response with transfer of 25 μg PCL-F, but not with 5 μg of PCL-F. In the other experiment another batch of PCL-F was employed and as little as 7.5 μg of PCL-F transferred the ability to elicit a significant ear swelling response.

Fig. 2 shows that the ear swelling response that PCL-F transferred was antigen specific. When PCL-F that was derived from lymphoid cells of mice that were contact sensitized with PCL was used, immediate reactions were elicited in one ear with PCL, but not in the contralateral ear with oxazolone. PCL-F produced in CBA mice was also able to transfer the ability to elicit specific immediate ear swelling responses in several other inbred strains of mice (BALB/C, C3H, BDF_1).

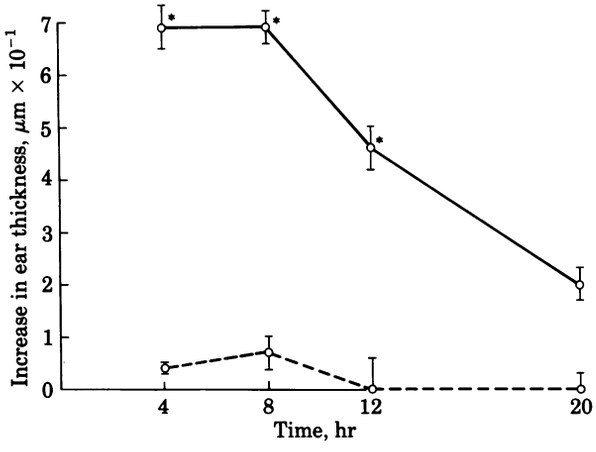

FIG. 2. Specificity of contact ear swelling reaction in mice receiving PCL-F. Five male CBA/J mice were injected i.v. with 60 μg of hapten affinity-purified PCL-F and were challenged immediately thereafter on the right ear with 0.8% PCL in olive oil (——) and on the left ear with oxazolone (----). Five noninjected controls were challenged similarly and their resulting ear swelling response to PCL and oxazolone at each time point was subtracted from the response of the mice that were injected i.v. Data are given ± SEM.
* Statistically significant difference ($P < 0.01$) compared to contralateral ear challenged with oxazolone or ears of noninjected controls challenged with PCL.

PCL-F Is Not Made by B Cells. To rule out the possibility that PCL-F was an antibody, mixed spleen and lymph node cells from PCL contact-sensitized mice were depleted of B cells by adherence to anti-Ig plates. Cultures of lymphoid cells depleted of B cells, and therefore T cell enriched, yielded an equivalent amount of PCL-F (as determined by protein content of antigen-binding material) compared to the nondepleted population. Table 1 shows that PCL-F from B-depleted cells was as potent as PCL-F from the mixed cell population in transferring the ability to elicit an immediate contact ear swelling response. Thus PCL-F was unlikely to be a B lymphocyte product.

PCL-F Does Not Express Any Antigenic Markers Found on Immunoglobulins. An ELISA direct binding immunoassay was used in an attempt to detect determinants of heavy and light chains of conventional immunoglobulins in PCL-F compared to polyclonal and monoclonal standards. Table 2 shows that no determinants of conventional immunoglobulins (κ, λ, mixed γ, γ1, γ2a, γ2b, μ, α, ε, δ) were detected in PCL-F by ELISA. Because the ELISA had a detection sensitivity of about 5–10

Table 1. Contact ear swelling reactions elicited with PCL in recipients of PCL-F derived from T cells

Cells used as source of PCL-F	1.5-hr ear-swelling response, μm × 10^{-1}
Whole spleen and lymph node	5.2 ± 0.5
T cells (B-depleted)	6.6 ± 1.3
None	0.8 ± 0.2

Spleen and lymph node cells from mice contact sensitized with PCL were harvested on day 4, and half the cells were depleted of B cells by adherence to anti-mouse Ig plates. Less than 3% of these cells stained for surface immunoglobulin. Supernatants from whole and B-depleted cells were harvested after 48 hr of culture and PCL-F derived from each population was purified and concentrated by affinity chromatography and transferred i.v. to groups of normal mice (three per group, 75 μg per mouse) that were challenged immediately thereafter with 0.8% PCL applied to their ears. Results are given as mean ± SEM.

Table 2. Survey of serological determinants associated with CBA/J-derived PCL-F

Reagents used with negative results
Rabbit reagents
Anti-κ (7), anti-λ (7), anti-α (5), anti-μ (12), anti-ε (7), anti-γ1 (3), anti-mixed γ (4)
Monoclonal antibodies
Anti-Ig5a (δ) (4)
Reagents used with positive results
Rabbit anti-TSF (12), rabbit anti-PCL-F (12), rabbit anti-rat T cell receptor (7)

Twelve different PCL-F preparations were assayed altogether. The number assayed for each determinant is given in parentheses. All assays were performed by ELISA direct binding using HRP conjugates of goat anti-rabbit Ig or goat anti-mouse Ig and *o*-phenylenediamine substrate for color development. Negative results were judged as color intensities obtained with a 1:20 dilution of a given antisera that were less than 2 × the color obtained with 1:20 nonimmune pooled rabbit serum. The positive titer estimates given below were based on color intensities at limiting dilutions that were at least 2 × blank (pooled nonimmune rabbit serum diluted 1:20). Often 1:10,000 was the last dilution employed and sera yielding color intensities in excess of 2 × blank are listed as titers of at least 1:10,000.

ELISA reagents employed and positive control ELISA titers: *Anti-*κ. Litton Bionetics; titer vs. MOPC 183 (IgM-κ) at least 1:10,000. *Anti-*λ. Gateway Immunoserum; titer vs. MOPC 104E (IgM-λ) at least 1:10,000. *Anti-*α. Gateway Immunoserum; titer vs. MOPC 460 (IgA) at least 1:1000. *Anti-*μ. Litton Bionetics; titer vs. MOPC 104E (IgM) at least 1:10,000. *Anti-*ε. Gift from David H. Katz and F.-T. Liu (15); titer vs. hapten affinity-purified monoclonal anti-dinitrophenyl IgE (ref. 15; also a gift from D. H. Katz and F.-T. Liu) at least 1:10,000. *Anti-*γ1. Litton Bionetics; titer vs. pooled mouse Ig at least 1:1000. *Anti-mixed* γ. Litton Bionetics, pool of anti-γ2a, γ2b, γ1, γ3; titer vs. pooled mouse Ig at least 1:1000. *Anti-*δ. Beckton, Dickinson, mouse hybridoma anti-Ig5a; this reagent stained determinants on B cells. *Anti-TSF*. R11, gift from Robert Cone (13) and R20 prepared similarly; titer vs. PCL-F 1:500; titer vs. mouse Ig standards was negative (13). *Anti-PCL-F*. R16, gift from Robert Cone; titer vs. PCL-F was 1:500; titer vs. mouse Ig standards was negative (prepared as in ref. 13). *Anti-rat T cell receptor* (serum 801). Gift from Hans Binz (16); titer vs. PCL-F > 1:100; titer vs. mouse Ig standards was negative (16).

ng/ml and the limiting dose of PCL-F for successful transfer was 7.5–25 μg, it was very unlikely that molecules containing determinants of conventional immunoglobulins were responsible for the transfer of contact ear swelling responses.

PCL-F Expresses Markers Found on Some T Cells and Their Antigen-Specific Molecular Mediators. In addition, the ELISA direct binding immunoassay showed that rabbit antisera to T cell-derived antigen-binding suppressor factor (TSF) (12, 13), rabbit anti-PCL-F, and rabbit anti-rat T cell receptor (16) all could bind to PCL-F (Table 2).

We can thus conclude that PCL-F is an antigen-binding T cell material, because (*i*) it does not contain immunoglobulin determinants; (*ii*) it is derived from lymphoid cells depleted of B cells; and (*iii*) it is recognized by antisera that bind other T cell factors.

PCL-F Is Considerably Smaller Than Immunoglobulins. PCL-F was fractionated according to size by a sucrose gradient. In the experiment shown in Table 3, and in three additional experiments, only the two fractions containing material in the molecular weight range of 45,000–70,000 were able to transfer the ability to elicit an immediate contact ear swelling response. IgE standard, included as a control, was not found in this portion of the gradient but was detected in its expected position according to the molecular weight standards (Table 3).

Table 3. Activity of sucrose gradient fractions of PCL-F in transferring the ability to elicit contact ear swelling responses

Sucrose gradient		Ear swelling response, μm × 10^{-1}	
Fraction	Marker (M_r)	At 1 hr	At 3 hr
1	IgM (900,000)	0.0 ± 0.5	0.7 ± 0.5
2	(Between 200,000 and 800,000)	0.7 ± 0.7	0.7 ± 0.7
3	TNP-BGG (150,000 to 200,000)	0.1 ± 0.6	0.8 ± 0.5
4	Albumin (70,000)	1.0 ± 0.4	3.1 ± 0.3
5	Ovalbumin (45,000)	4.7 ± 0.3	5.5 ± 0.9

About 200 μg of PCL-F was fractionated by centrifugation through a sucrose gradient and a duplicate gradient was run with the markers indicated. Fractions were dialyzed into normal saline and were transferred i.v. into normal recipients (four per group) that were challenged immediately thereafter by topical application of 0.8% PCL in olive oil. A separate group of noninjected controls was challenged similarly and their resulting ear swelling response at 1 or 3 hr was subtracted from the responses of mice that received PCL-F. Results are given ± SEM. The peak of IgE standard was detected by ELISA in fraction 3, some was also found in fraction 4, and none was found in fraction 5.

DISCUSSION

Various T cell-derived factors are involved in mediating the events required for the elicitation of contact sensitivity and other delayed-type hypersensitivity responses. These factors are essential for recruiting inflammatory cells into tissue spaces where foreign antigens are introduced. Many of these are non-antigen-specific factors (lymphokines) that serve to recruit circulating bone marrow-derived cells to the reaction site through chemotactic and migration-inhibiting activities (17). Our previous work had suggested that amongst the potentially numerous T cell factors that must be released for elicitation of delayed-type hypersensitivity reactions was a factor that led to mast cell release of vasoactive amines (see Introduction and refs. 4–6).

The results presented demonstrate that T cells from mice that are immunized in a way that is optimal for eliciting contact hypersensitivity responses can make a product that can cause a very rapid swelling at the skin site where the specific contactant is applied. The antigen-binding factor that mediates the rapid contact skin reactions was shown to be T cell derived and not an antibody by four criteria: (*i*) B cell depletion did not affect production; (*ii*) no immunoglobulin determinants were detected; (*iii*) the molecular weight of about 70,000 or less is clearly below that of any known immunoglobulin molecule; and (*iv*) determinants on this T cell factor were recognized by antisera that can bind other antigen-specific T cell factors, or some T cells themselves, and do not bind to immunoglobulin (12, 13, 16). Thus the T cell-released material that mediated immediate hypersensitivity-like reactions is unrelated to any known B cell product and shares markers with other biologically active T cell products. Although the T cell factor appears to be antigen specific, study of similar factors derived from donors immunized with other antigens will be necessary to rule out the possibility that our findings are peculiar to sensitization or elicitation (18) with the antigen we have employed.

The most likely explanation for our findings is that the T cell-released factor arms vasoactive amine-carrying mast cells that can then be triggered by specific antigen, or the factor sensitizes other cells to release non-antigen-specific factors that activate mast cells to release vasoactive amines. Further study of this T cell factor will be necessary to establish that its function is indeed mast cell dependent and whether this factor accounts for T cell activation of mast cells in delayed-type and contact reactions, and thus functions *in vivo* at critical early steps in cell-mediated hypersensitivity.

The reason why this apparently important T cell-mediated function has previously escaped detection could be threefold:

(*i*) Under physiological conditions only small amounts of the material are made. Using hapten affinity chromatography, we were able to purify and concentrate antigen-binding material derived from bulk cultures of lymphoid cells of large groups of mice. The amount of material we employed for systematic transfers in individual mice was derived from the combined lymph node and spleen cells of 1–2 donors per recipient. Thus the technique we have used may result in the production of significantly more material than is usually made *in vivo*. (*ii*) It is likely that the synthesis of the T cell factor during *in vivo* responses is confined to the sites where the antigen is placed and it is probably not released in significant amounts into the circulation. It is possible that some low levels of the material do circulate but have not been previously identified because of a lack of reagents required for identification. (*iii*) The T cell-released mast cell activating factor may have a much shorter half-life than the material made by IgE^+ B cells.

We should consider why there is no immediate reaction in cases in which mice are optimally immunized for delayed-type hypersensitivity and presumably can make the factor we have described and also do not have IgE. We suggest that under physiological conditions the T cells that release the factor that leads to mast cell activation require a significant period of time to reach the site where the antigen is located and then interact with the antigen and release their product. Thus, under normal circumstances one would not necessarily find a significant immediate sensitivity portion of a physiological delayed-type hypersensitivity response. However, one can concentrate one of the factors that take part in these reactions and use it in excess concentration to produce an immediate contact ear swelling response. Thus in the overall picture, local and delayed production in small amounts and short half-life may combine to make the final vasoactive effects of the T cell factor subtle compared to those that are produced by IgE. However, our results indicate that even though this factor may be made in small amounts and may be rapidly degraded, its activity appears to be as essential for the elicitation of delayed-type hypersensitivity reactions as is IgE for the elicitation of standard immediate hypersensitivity reactions.

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