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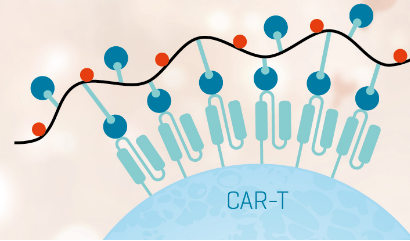

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J Immunol (1980) 125 (6): 2790–2796.
https://doi.org/10.4049/jimmunol.125.6.2790

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0022-1767/80/1256-2790$02.00/0
THE JOURNAL OF IMMUNOLOGY
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Vol. 125, No. 6, December 1980
Printed in U.S.A.

STUDIES ON STIMULATION OF CELL-MEDIATED CYTOTOXICITY BY SKIN TEST ANTIGENS

I. Candida Antigen Stimulation of Cell-Mediated Cytotoxicity *in Vitro* Correlated with the Skin Test Response to Candida Antigen *in Vivo*[1]

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The magnitude of the skin test response in a group of normal volunteers to intradermal Candida antigen correlated closely with the level of cytolytic activity stimulated by Candida antigen *in vitro* in the peripheral blood lymphocytes from those same individuals. The cytolytic activity stimulated by Candida antigen was cell mediated, and cold target inhibition studies demonstrated that Candida antigen-stimulated effector cells were capable of cross-species (i.e., nonspecific) killing. Analysis of the Candida antigen-induced effector cell population for various cell markers did not enable absolute identification of the cell responsible for the killing. The findings in this study indicate that the stimulation of cell-mediated cytolysis by Candida antigen may be related to the delayed-type hypersensitivity response produced by the same antigen.

Antigens such as Candida antigen and purified protein derivative (PPD), which may induce a delayed-type hypersensitivity (DTH)[3] response *in vivo* in certain individuals, have also been shown to stimulate a lymphocytic proliferative response *in vitro* (1). Previous observers noted that exposure of human peripheral blood lymphocytes (PBL) to these antigens *in vitro* may also result in the generation of cytolytic effector cell activity against various ^{51}Cr-labeled target cells (2–4). One study showed that PBL from a tuberculin-positive donor exposed to PPD *in vitro* developed a higher level of cytotoxicity toward a Chang human liver cell target than did similar cells from a tuberculin-negative donor (2). Similar results were found when PBL from 3 human volunteers, 2 tuberculin-positive and 1 tuberculin-negative, were stimulated with PPD *in vitro* and tested against August rat hepatoma target cells (4). Another report dealing with immune responsiveness in patients with chronic candidiasis demonstrated that exposure of PBL from a group of these patients to Candida antigen *in vitro* resulted in lower levels of cytolytic activity against ^{51}Cr-labeled chicken red blood cells than did similar exposure of PBL from normal individuals (3).

Since these studies demonstrated there might be an association between the stimulation of DTH *in vivo* and the generation of cell-mediated cytolysis (CMC) *in vitro* by certain antigens, we decided to further investigate this interesting possibility using Candida antigen. We found that the magnitude of an individual's response to Candida antigen on skin testing correlated highly with the level of CMC generated *in vitro*. Cold target inhibition assays indicated that the CMC generated after exposure to Candida antigen resembled that expressed by natural killer (NK) cells (5–8).

MATERIALS AND METHODS

Target cells. P815 mastocytoma cells were originally isolated from DBA/2 (H-2^d) mice and were maintained in culture in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (FCS; Flow Laboratories, Rockville, MD). Both the Daudi and Raji lymphoma cell lines were originally isolated from patients with Burkitt's lymphoma and were maintained in culture in RPMI 1640 supplemented with 10% heat-inactivated FCS. Labeling with ^{51}Cr was done as described by Brunner *et al.* (9).

Reagents. Allergenic extracts of *Candida albicans* (Hollister-Steir Laboratories, Downers Grove, IL) were dialyzed against 3 changes of sterile phosphate-buffered saline (PBS), filtered through a 0.2-μ Nalgene filter (Nalge/Sylron Corp., Rochester, NY), pooled, and stored at −20°C. Concanavalin A (Con A; Pharmacia Fine Chemicals, Uppsala, Sweden) was dissolved in PBS at 1.0 μg/ml and stored at −70°C.

Isolation of human PBL. Venous blood from normal human volunteers was collected in a sterile manner using EDTA[3] as anticoagulant. PBL were isolated utilizing Ficoll-Hypaque gradients. Human PBL preparations obtained in this manner from normal volunteers contained from 60 to 75% lymphocytes with the remaining cells monocytes.

Stimulation and culture of human PBL. Cultures were established by placing 20 × 10^6 viable (i.e., trypan blue-excluding) cells from the separation above in 10 ml of culture medium

Received for publication May 28, 1980.
Accepted for publication September 2, 1980.
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[1] This work was supported by a research grant from the Leukemia Research Foundation and by United States Public Health Service Grants CA 19266 and CA 14364 from the National Cancer Institute.
[2] Supported in part by Pathology Departmental Training Grant GM00093.

[3] Abbreviations used in this paper: PBL, peripheral blood lymphocytes; CMC, cell-mediated cytolysis; DTH, delayed-type hypersensitivity; NK, natural killer; DMEM, Dulbecco's modified Eagle's medium; EBSS, Earle's balanced salt solution; HABS, human AB+ serum; SKSD, streptokinase/streptodornase; EDTA, ethylenediaminetetraacetic acid; MOPS, morpholinopropanesulfonic acid; C:T ratio, number of cells originally cultured:number of target cells; E:T ratio, effector cells:target cells.

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consisting of RPMI 1640 supplemented with 5% heat-inactivated pooled AB+ serum (HABS) and 10 mm morpholinopropanesulfonic acid (MOPS) in 50-ml tissue culture flasks (Falcon No. 3013, Oxnard, CA). MOPS is an organic buffer used to maintain a pH of 7.2 during culture.

Assay for cell-mediated cytotoxicity. At the times indicated, the cells remaining in each culture flask were harvested, washed, and resuspended in 2 ml assay medium, which consisted of Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% heat-inactivated FCS. Serial 1/3 dilutions of the cells were made, and 0.1 ml of each dilution was added to 2 round-bottom microtiter wells (Linbro Microliter "U" plate, Hamden, CT). Each well then received 5×10^3 ^{51}Cr-labeled target cells in 0.1 ml of assay medium. The assay plates were then centrifuged for 2 min at 200 × G and incubated for 4 hr at 37°C in a humidified atmosphere containing 5% CO_2 in air. At the end of this time, the plates were spun for 5 min at 500 × G, and 0.1 ml of supernatant fluid was removed from each well for counting in a well-type scintillation counter (Nuclear Chicago-Searle, Des Plaines, IL). The percentage of cytotoxicity for each dilution was calculated from the expression:

$$cpm_{exp} - cpm_{spont.\ release}/cpm_{max} - cpm_{spont.\ release} \times 100.$$

Maximal release was determined by freezing and thawing of the target cells. Since the number of cells in each dilution was known, it was possible to construct dose-response curves. By interpolation from these curves, lytic activity could be determined both for the number of cells originally placed in culture and for the number of cells placed in assay. The lytic activity developed by a given number of cells originally placed in culture was determined by calculating the percent of target cells lysed by a culture fraction that had contained that number of cells at the start of culture several days previously. This was expressed as the percent lysis developed at various C:T (number of cells originally cultured:number of target cells) ratios. Thus, the percent lysis of a C:T ratio of 100:1 represents the percent of 5×10^3 target cells lysed by a culture fraction that had contained 5×10^5 PBL at the initiation of culture. Lytic activity per number of cells actually placed in assay was expressed as the percent lysis developed at various E:T (number of cells in the effector cell population assayed:number of target cells) ratios.

Inhibition of cytotoxicity. The cells remaining after 4 days of exposure of human PBL to a concentration of Candida antigen optimal for inducing cytolytic effector cell activity were placed in cytolytic assays together with 5×10^3 ^{51}Cr-labeled P815 mouse mastocytoma target cells at an E:T ratio of 100:1. Various unlabeled target cells were then added to the wells containing the cytolytic effector cells and ^{51}Cr-labeled P815 target cells at 2.5:1, 5:1, and 10:1 unlabeled:labeled target cell ratios. The assay plates were then centrifuged for 2 min at 200 × G and incubated 4 hr at 37°C in a humidified atmosphere containing 5% CO_2 in air. Lytic activity was determined as described previously. The percent inhibition was calculated by the formula:

$$\frac{\%\ lysis_{(-)\ Unlabeled\ targets} - \%\ lysis_{(+)\ Unlabeled\ targets}}{\%\ lysis_{(-)\ Unlabeled\ targets}} \times 100.$$

Characterization of mononuclear cells. Cells were analyzed for various markers according to the following procedures:

Peroxidase staining. The percent of cells positive for blue staining with peroxidase reagent was determined. Monocytes and granulocytes are peroxidase positive, whereas lymphocytes are peroxidase negative.

E-rosette assay. To 50 μl of mononuclear cells at 1×10^7 cells/ml were added 50 μl of FCS and 100 μl of 2.5% SRBC. The mixture was incubated 5 min at 37°C, centrifuged 5 min at 200 × G, and incubated an additional 2 hr at 4°C. After incubation, the cells were gently resuspended in the cold, and the percent of cells that were morphologically lymphocytic with 3 or more SRBC attached was determined. The ability to form E-rosettes under these conditions is a unique property of human T lymphocytes.

EAC rosette assay. To 100 ml of mononuclear cells at 1×10^7 cells/ml in Earle's balanced salt solution (EBSS) were added 10 μl of EDTA at 150 μg/ml. After incubation for 30 min at 37°C, 25 μl of SRBC previously treated with rabbit anti-SRBC IgM and C5-deficient mouse serum were added. A separate aliquot of the cells was similarly incubated with SRBC treated with rabbit anti-SRBC IgM alone as a control. The tubes were then incubated 15 min on a rotor at 37°C, after which time the percent of cells morphologically lymphocytic with 3 or more SRBC attached was determined. Lymphocytes forming EAC rosettes under these conditions bear receptors for complement (C).

EA-rosette assay. To 50 μl of mononuclear cells at 1×10^7 cells/ml in RPMI were added 50 μl of SRBC previously treated with rabbit anti-SRBC IgG and 25 μl of FCS. The mixture was then centrifuged at 200 × G for 8 min, followed by incubation for 45 min at 37°C. After incubation, the cells were gently resuspended and the percent of lymphocytes with 3 or more SRBC attached was determined. Lymphocytes forming EA rosettes under these conditions bear receptors for the Fc portion of IgG.

Assay for surface immunoglobulin (Ig). After incubating the cells in fresh medium for 1 hr at 37°C to remove any cytophilic antibody, the cells were treated wtih fluorescein-conjugated polyvalent goat anti-human Ig, and the percent of lymphocytes bearing the fluorescein stain was determined using a fluorescent microscope. Lymphocytes stained in this manner are B cells.

RESULTS

Stimulation of cytolytic activity with soluble Candida antigen. In order to examine the time course for the generation of CMC with Candida antigen, human PBL were isolated from 2 donors and exposed to a concentration of Candida antigen known to be optimally mitogenic. On succeeding days, the cells were examined for cytolytic activity against both a ^{51}Cr-labeled mouse DBA/2 (H-2^d) mastocytoma target cell and a similarly labeled human Burkitt's lymphoma-derived Daudi cell target. The lytic activity developed was calculated both for the number of cells originally cultured and for the actual number of cells assayed. Figure 1 shows the results from one such experiment. Here it can be seen that the cells from the 2 donors displayed cytolytic activity against both targets when assayed on day 0. On subsequent days, the unstimulated cells from both donors showed a loss in activity against the mouse mastocytoma target cell and a very slight increase in activity against the human Daudi cell target. In contrast, there was increased cytolytic activity against both target cells in the cells from both donors exposed to Candida antigen during culture. Since the cells exposed to Candida antigen showed lower levels of cytolytic activity against the mouse mastocytoma target cell than against the human Daudi cell target, cytolytic activity toward the mouse mastocytoma target cell is shown at both 30:1 and 100:1 E:T ratios. Similar curves were obtained when the data were plotted at 30:1 and 100:1 C:T ratios (i.e., per given number of cells originally cultured rather than per given number of cells from the effector cell population placed in assay). Lytic activity

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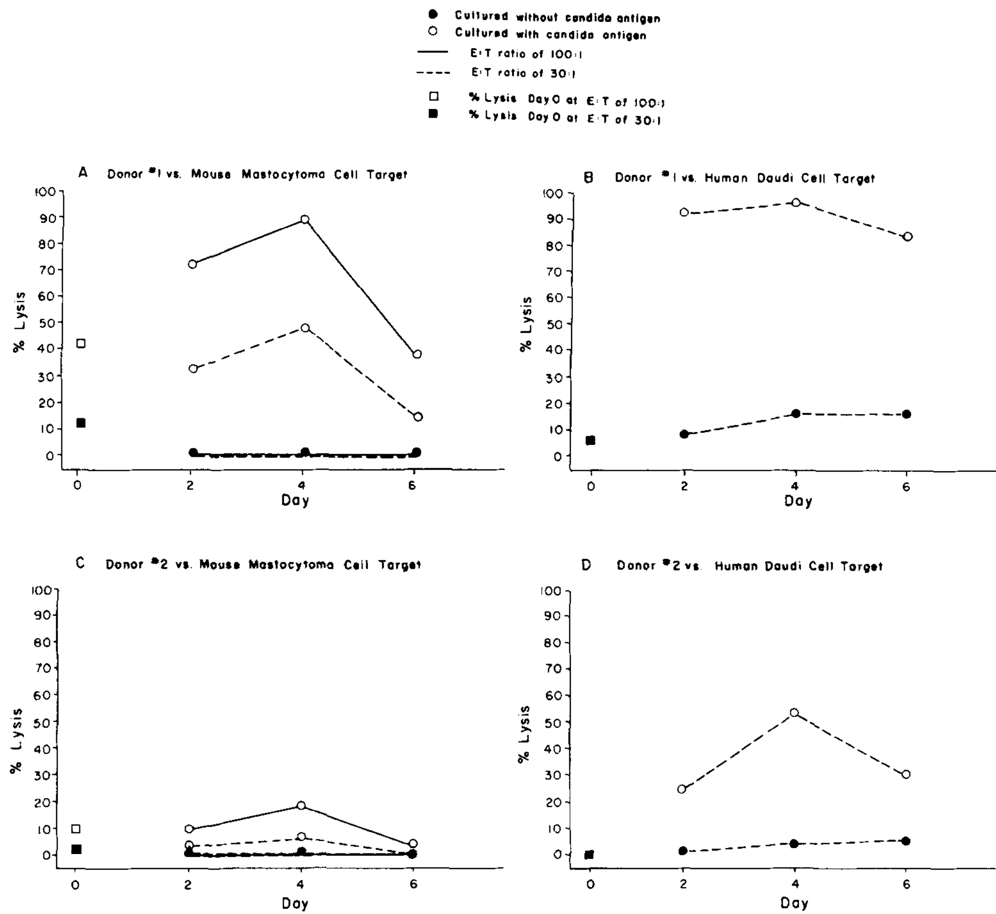

Figure 1. Cytolytic activity stimulated in human PBL exposed to soluble Candida antigen *in vitro*. Human PBL were isolated from 2 normal volunteers as described in *Materials and Methods.* A portion of the cells were tested for cytolytic activity against both P815 mouse mastocytoma and human Daudi cell targets. The remainder of the cells were cultured in the presence and absence of 1:10 Candida antigen as described in *Materials and Methods.* Cells were removed from culture on days 2, 4, and 6 and tested for cytolytic activity against the mouse mastocytoma and human Daudi cell targets. Lytic activity was determined for the number of cells assayed (*E*) as described in *Materials and Methods.* Panels A and B show the lytic activity of the cells from the first donor against both the mouse (*Panel A*) and human (*Panel B*) target cells after various periods of culture. *Panels C* and *D* show the lytic activity of the cells from the second donor against the same targets under identical conditions.

against both target cells was maximal at or near day 4 in the Candida antigen-stimulated cultures in the cells from both donors, although the level of cytolytic activity against both target cells was considerably higher in those from donor 1 than in those from donor 2. Thus, Candida antigen clearly generated increased levels of cytolytic activity in human PBL against target cells from both species. The mechanism accounting for the apparent nonspecificity of the Candida antigen-induced cytolysis was as yet unclear. One possibility was that Candida antigen might have stimulated certain cells to release a cytotoxic factor into the medium that possessed the ability to nonspecifically lyse various cell lines. We therefore conducted experiments to explore this potentiality.

Experiment to determine whether Candida antigen-stimulated effector cells release a cytotoxic factor into the medium. In order to ascertain whether Candida antigen stimulated the release of a nonspecific cytolytic factor into the medium, ^{51}Cr-labeled Daudi target cells were incubated in the presence of supernatants taken immediately from assays where Candida-stimulated cytolytic effector cells had previously lysed unlabeled Daudi target cells and also from assays where the effector cells had been cultured alone. As seen in Table I, the supernatants had no effect on the ^{51}Cr-labeled Daudi target cells, making it highly unlikely that a cytotoxic factor released into the medium was responsible for the cross-species killing displayed by the Candida antigen-stimulated effector cells. Another possible explanation for the diversity of the Candida antigen-stimulated cytolysis was that residual Candida antigen might enable effector cells to bind and nonspecifically lyse a wide variety of target cells. Since the plant lectin Con A had been shown to mediate this type of nonspecific binding and cytolysis (10, 11), it was decided to see whether Candida antigen might possess similar properties.

Experiments to determine whether Candida antigen enables nonspecific cytolysis. In order to determine whether soluble Candida antigen might possess the ability to enable nonspecific cytolysis of a target cell by human effector cells, human PBL stimulated *in vitro* with Con A were placed in replicate cytolytic assays with various concentrations of Candida antigen or a concentration of Con A known to mediate nonspecific cytolysis. Table II shows the results from an experiment using ^{51}Cr-labeled mouse mastocytoma target cells and Con A-stimulated human effector cells from 2 different donors. In the presence of medium alone (i.e., without Con A or Candida antigen added to the assay medium), there was less than 10% cytolysis of the mouse target cell by the Con A-stimulated effector cells from either donor. The cytolysis of that target cell was greatly enhanced in the assays containing 15 μg/ml Con A, however, there was no similar enhancement in the presence of various concentrations of Candida antigen. Another experiment to see

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TABLE I

Experiment to determine if Candida antigen-stimulated effector cells release a cytotoxic factor into the medium[a]

Part A: % Lysis of ^{51}Cr-labeled Daudi Target Cells by Candida Antigen-Stimulated Effector Cells		Part B[b]: % Lysis of ^{51}Cr-labeled Daudi Target Cells by Supernatants Taken from Previous Assays		
E:T ratio	% lysis	Concentration of supernatant	Supernatant from	
			E + T	E alone
		%	*% lysis*	
100.0:1	99.6	0	0	0
33.0:1	99.9	1	0	0
11.0:1	83.5	5	0	0
3.7:1	51.4	10	0	0
1.2:1	19.3	25	0	0
		50	0	0
		75	0	0
		90	0	0

[a] PBL were exposed to Candida antigen at a concentration of 1:10 for 4 days after which the remaining cells were harvested, washed, and placed in simultaneous assays with: ^{51}Cr-labeled Daudi cell targets at the E:T ratios shown (Part A), unlabeled Daudi cells at 100:1 E:T ratio (5×10^5 effector cells + 5×10^3 unlabeled target cells), and in assay trays alone at 5×10^5 effector cells per well. The assay trays were then incubated for 4 hr in a humidified atmosphere with 5% CO_2 in air. At the end of this time, the supernatants were collected. Lytic activity was determined as described in *Materials and Methods*.

[b] In Part B the supernatants from the wells that had contained the effector cells plus the unlabeled target cells (E + T) and the wells that had contained the effector cells alone (E alone) were collected and added to wells containing 5×10^3 ^{51}Cr-labeled Daudi target cells at the concentrations shown. The assay trays were then incubated 4 hr in a humidified atmosphere with 5% CO_2 in air and analyzed for lytic activity at the end of this time.

TABLE II

Compared effect on cytolysis between adding Candida antigen or Con A to assays containing mouse P815 target cells and human Con A-stimulated blast cells[a]

Agent Added to Cytolytic Assay		Source of Con A Blasts	
		D. T.	R. M.
		% lysis	
Medium alone		9	9
Candida antigen	1:20	9	9
	1:200	9	9
	1:1,000	9	9
	1:10,000	9	9
Con A	15 μg/ml	68	47

[a] PBL isolated from 2 donors (D. T. and R. M.) were cultured for 3 days in the presence of 10 μg/ml Con A as described in *Materials and Methods*. At the end of that time the cells were harvested and placed in replicate cytolytic assays with ^{51}Cr-labeled mouse P815 mastocytoma target cells at an E:T ratio of 100:1. Various concentrations of Candida antigen, medium alone, or a concentration of Con A known to mediate nonspecific cytolysis were included at the initiation of assay. The assays were incubated for 4 hr and cytolytic activity was determined as described in *Materials and Methods*.

whether Candida antigen in the assay might affect cytolysis was performed by adding various concentrations of the antigen to identical cytolytic assays containing Candida antigen-induced effector cells and ^{51}Cr-labeled mouse mastocytoma target cells. As seen in Table III, the various concentrations of Candida antigen added to such assays did not appreciably affect cytolytic activity. In view of these results, it was unlikely that small amounts of Candida antigen bound to the cells could mediate nonspecific cytolysis. These studies indicated that Candida antigen could, in fact, stimulate high levels of CMC against cells from different species. The question remained, however, whether the CMC generated by Candida antigen involved individual effector cells capable of cross-species killing or whether the antigen might have stimulated a wide variety of effector cells bearing different specificities. Since previous investigators had demonstrated the usefulness of cold target inhibition assays in detecting the presence of "nonspecific" cytolytic effector cells (12), we decided to utilize this technique to see whether the Candida antigen-induced effector cells possessed this capability.

Cold target inhibition of Candida-induced effector cells. The experiment illustrated in Figure 2 was performed to see how the addition of graded numbers of various unlabeled human target cells would affect cytolysis of a ^{51}Cr-labeled mouse target cell by human Candida antigen-generated effector cells. Here it can be seen that both the human Daudi and human Raji unlabeled target cells inhibited cytolysis of the mouse mastocytoma target cell by the human Candida antigen-generated effector cells (49 and 51%, respectively, at 10:1 unlabeled: labeled target cell ratio). The inhibition was not due to crowding, since similar numbers of Con A-stimulated syngeneic and allogeneic human blast cells produced much less inhibition (6% and 5%, respectively, at a 10:1 unlabeled:labeled target cell ratio). Similar results were obtained when the reverse combination was tested, i.e., when unlabeled mouse mastocytoma target cells were used to inhibit lysis of ^{51}Cr-labeled human Daudi cell targets by Candida antigen-generated human effector cells (data not shown). These results demonstrated that the effector cells generated after exposure of PBL to Candida antigen were capable of cross-species (i.e., nonspecific) killing. As illustrated in Figure 1, we had noted on numerous occasions that PBL from various individuals developed different levels of CMC after exposure to Candida antigen. We decided next, therefore, to analyze the question of whether there might be some connection between the antigenic stimulation of CMC and the response to that antigen on skin testing.

Candida antigen-stimulated CMC and skin test responsiveness compared in a group of normal volunteers. In order to compare antigenic stimulation of CMC *in vitro* with the DTH response *in vivo*, PBL were isolated from 5 donors and exposed in culture to an optimally mitogenic concentration of Candida

TABLE III

Effect of adding various concentrations of Candida antigen to ongoing cytolytic assays containing Candida antigen-stimulated human effector cells and ^{51}Cr-labeled mouse mastocytoma target cells[a]

		E:T	
		30:1	100:1
		% lysis[b]	
Medium alone		25	59
Candida antigen	1:10	25	56
	1:100	30	64
	1:1,000	28	61
	1:10,000	27	61

[a] PBL were exposed to Candida antigen at a concentration of 1:10 for 4 days after which the remaining cells in culture were harvested, washed, and placed in identical cytolytic assays with ^{51}Cr-labeled mouse mastocytoma cells at the E:T ratios shown. At the initiation of the assays, Candida antigen was included at the concentrations shown. The assays were then incubated for 4 hr in a humidified atmosphere containing 5% CO_2 in air and lytic activity was determined.

[b] Percent lysis was calculated as described in *Materials and Methods*.

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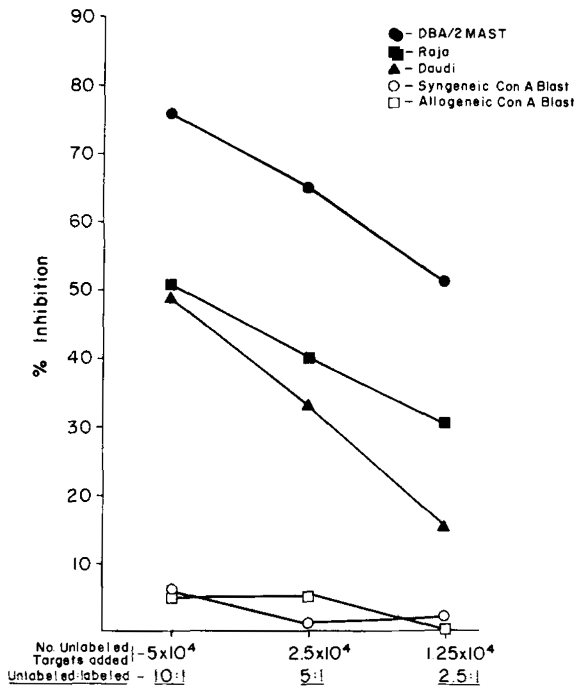

Figure 2. Cold target inhibition of Candida antigen-stimulated cytolytic effector cells. Human PBL were cultured with soluble Candida antigen, final concentration 1:10, as described in *Materials and Methods*. After 4 days the cells were harvested, washed, and placed in replicate CTL assays with ^{51}Cr-labeled mouse P815 mastocytoma target cells at an E:T ratio of 100:1. Various cold (*unlabeled*) target cells were then added to the assays at, *Unlabeled:Labeled*, target ratios of 2.5:1, 5:1, and 10:1. Cytolytic activity and percent inhibition of cytolytic activity were determined as described in *Materials and Methods*.

antigen. Immediately after obtaining blood, 0.2 ml of Candida antigen was applied intradermally to the right forearm of the same individuals. The erythema and induration that developed in the area of the intradermal injection was measured after 48 hr. Cells from the Candida antigen-stimulated cultures were tested for cytolytic activity against both mouse mastocytoma and human Daudi cell targets after 4 days of culture. The results are shown in Table IV. Correlation coefficients were determined between the various responses, and these are shown in Table V. The correlation coefficient (r) is a measure of how well one set of data correlates with another set. A value close to 1.0 indicates a high correlation close to 0 indicates the results were unrelated. For sets of data in 5 individuals (n = 5), a correlation coefficient of 0.805 or greater between 2 sets of findings gives a confidence level of greater than 90% that those results are related. As expected, there was a very high correlation (r of 0.98 and 0.99) between the 2 methods of expressing lytic activity (i.e., C:T *vs* E:T). There was also a high degree of correlation between the lytic activity developed against the human Daudi cell target cell and that shown against the human Daudi cell target after Candida antigen stimulation regardless of whether the lytic activity was expressed in terms of the number of cells originally cultured or the number of cells placed in assay (i.e., expressed at a given C:T or E:T ratio). The Candida antigen-stimulated cytolytic activity in the PBL from the various individuals correlated highly (r of 0.80 to 0.96) with the skin test responses (erythema and induration) at 48 hr in those same individuals. Significantly, there was no apparent correlation (r less than 0.5) between the residual lytic activity in the unstimulated control cultures and any of the Candida antigen-stimulated responses (data not shown). From these results, it was apparent that in these normal individuals there was a correlation between Candida antigen stimulation of cytolytic activity *in vitro* and skin test reactivity *in vivo*. Next, we decided to analyze the cells harvested after 4 days of exposure of PBL to Candida antigen for the presence of various cell markers.

Analysis of the Candida antigen-induced effector cell population for various cell types. The cells harvested after 4 days of exposure of human PBL to a concentration of Candida antigen optimal for inducing CMC were analyzed for the presence of peroxidase staining, Fc receptors, C receptors, SRBC receptors, and surface Ig. The results from 3 separate experiments are shown in Table VI, where it can be seen that very few of the lymphocytes that remained after stimulation with Candida antigen formed EAC rosettes (1.5 to 7.5%), formed EA rosettes (1.5 and 2.0%), or bore Ig on their surface. Also, very few of the cells harvested were peroxidase positive (3.5 to 8.5%). By far, the largest number of cells that could be identified via cell markers were lymphocytes that formed rosettes with SRBC (36:0 to 65.0%). Interestingly, a large proportion of the cells remaining after Candida antigen stimulation did not appear to

TABLE IV

Stimulation of skin test responsiveness and cytolytic activity by Candida antigen compared in a group of normal volunteers[a]

Donor	Skin Test Day 2[b]		Lytic Activity, Day 4[c]			
			P815-Target		Daudi-Target	
	Erythema	Induration	(100:1) C:T	(100:1) E:T	(30:1) C:T	(30:1) E:T
	mm					
DT	59	46	39 (0)	45 (0)	68 (5)	72 (7)
AM	26	12	24 (2)	34 (4)	59 (8)	63 (14)
KR	11	8	8 (0)	14 (0)	28 (7)	42 (11)
CDM	11	5	3 (0)	6 (0)	22 (3)	31 (6)
JE	10	8	4 (0)	5 (0)	27 (10)	31 (13)

[a] After peripheral blood was obtained from the various donors for isolation of PBL, 0.2 ml of Candida antigen extract was applied intradermally. PBL were placed in culture in the presence and absence of soluble Candida antigen (final concentration 1:10) as described in *Materials and Methods*. The values in parentheses represent the activity found in the unstimulated (without Candida antigen) cultures.

[b] The erythema and induration occurring after 48 hr at the site of intradermal injection of Candida antigen extract.

[c] Lytic activity developed *in vitro* on day 4 was determined by using both mouse P815 mastocytoma target cell and human Daudi lymphoma target cells as described in *Materials and Methods*. The percent lysis occurring at C:T and E:T ratios of 30:1 was used for the human Daudi cell target, whereas the percent lysis of occurring at C:T and E:T ratios of 100:1 was used for the mouse P815 target cell.

TABLE V

Correlations between Candida antigen-induced skin test reactivity and cytolytic activity in a group of normal volunteers[a]

			Lytic Activity-Day 4			
			P815-Target		Daudi-Target	
			(100:1) C:T	(100:1) E:T	(30:1) C:T	(30:1) E:T
Skin test	Erythema		0.96	0.91	0.89	0.89
Day 2	Induration		0.90	0.83	0.81	0.80
Lytic activity	Daudi	C:T (30:1)	0.98			0.98
		E:T (30:1)		0.99		
Day 4	P815	E:T (100:1)	0.99			

[a] Correlation coefficients were determined between sets of Candida antigen-stimulated responses by using the data shown in Table III.

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TABLE VI

Characterization of cells remaining after 4 days of exposure of human PBL to Candida antigen in vitro[a]

	Expt. 1	Expt. 2	Expt. 3
% Lysis[b]			
Daudi (30:1-E:T)	83.0	89.0	79.0
Raji (30:1-E:T)		77.0	
P815 (100:1-E:T)	37.0		83.0
% Cells bearing various cell markers[c]			
Peroxidase (+)	8.5	4.5	3.5
Lymphocytes forming E-rosettes	65.0	36.0	42.0
Lymphocytes forming EA-rosettes	2.0	1.5	
Lymphocytes forming EAC-rosettes[d]	7.5	3.0	3.0
Lymphocytes bearing surface Ig			1.0

[a] Human PBL were isolated and exposed to Candida antigen (final concentration 1:10) as described in *Materials and Methods*. On day 4 of culture the cells were assayed both for lytic activity and the presence of various cell markers. Experiments 1, 2, and 3 show the results by using PBL from the same individual on 3 separate occasions.

[b] Lytic activity was determined against the human Daudi and Raji cell targets and against the mouse P815 mastocytoma cell target at the E:T ratios indicated in a 4-hr ^{51}Cr-release assay with the protocol described in *Materials and Methods*.

[c] The percent of cells remaining bearing the various cell markers after 4 days of culture were determined by using the protocols described in *Materials and Methods*.

[d] There was no rosette formation in control assays where the SRBC had been pretreated with rabbit anti-SRBC IgM alone (i.e., in the absence of C).

bear any of the cell markers we were able to test for. These cells which were peroxidase negative, appeared similar in morphology to lymphocytes, but lacked surface Ig and did not form E, EAC, or EA rosettes.

DISCUSSION

These studies demonstrate that when PBL from a given individual are exposed to soluble Candida antigen for several days *in vitro*, they develop a level of CMC that correlates with the magnitude of the DTH response to Candida antigen on skin testing in the same individual. The CMC generated on exposure of human PBL to Candida antigen is reminiscent of that displayed by the previously described NK cell (5–8). Although "natural killing" was initially described as the spontaneous CMC found in PBL isolated directly from the circulation of various animals, including man (5–8), it has become clear that NK-like CMC can be generated or increased in human PBL by various means. Exposure of human PBL to stimuli such as alloantigens in mixed lymphocyte culture (13, 14), supernatants from PHA-stimulated lymphocytes (15), and interferon (16–18) also results in the generation of NK-like CMC. It is interesting in this regard that an antigen known to elicit DTH also stimulates increased NK-like CMC in human PBL. In postulating a mechanism to explain this observation, it may be necessary to consider certain findings by previous investigators. Over 10 yr ago it was observed that PBL from tuberculin skin test-positive donors exposed to PDD *in vitro* produced high levels of interferon, whereas similar cells from tuberculin skin test-negative donors did not (19). Recently, it was shown that interferon greatly enhance NK cell activity (16–18). Thus, the amount of interferon produced after exposure of individual's PBL to an antigen such as Candida antigen may depend on that person's sensitivity to that antigen, and the level of NK-like CMC generated may depend on the amount of interferon produced. This sequence of events would explain why there is specificity during Candida antigen stimulation of CMC that is not present during killing. Although the level of killing stimulated by Candida antigen reflected the sensitivity on skin testing of that donor to that antigen, the specificity of killing did not appear related to the original stimulus. Also, Candida antigen added to the cytolytic assay did not affect killing of the ^{51}Cr-labeled target cell, and we have observed that a variety of skin test antigens, including PPD and streptokinase/streptodornase (SKSD), can stimulate identical NK-like CMC in individuals sensitive to those antigens (data not shown). Proof that Candida antigen stimulation of NK-like CMC in human PBL depends on the production of interferon as a signal between a cell specific for that antigen and another cell that mediates the killing must await completion of further studies, which are planned.

Although it is clear from the present study that exposure of human PBL to a skin test antigen such as Candida antigen can result in the production of greatly increased levels of NK-like CMC, the origin and nature of the killer cell is as yet unknown. Exposure to Candida antigen might stimulate NK cells to divide, resulting in a quantitative increase of NK activity and/or such exposure could lead to a qualitative increase in killing via "enhancement" of CMC by NK cells, similar to what has been shown to occur when NK cells are directly exposed to interferon (16–18). Also, there may be actual induction of new killer cells from a subset of noncytolytic precursors. Experiments are underway to help differentiate between these various possibilities. In order to characterize the cell responsible for the Candida antigen-stimulated CMC, various separation procedures were attempted; however, purity of separation and questions concerning the cell marker analyses made it difficult to achieve satisfactory results. As seen in this study, many of lymphocytes present after Candida antigen exposure did not appear to have Ig or receptors for SRBC, the Fc portion of IgG or C on their surface. At the present time, we cannot exclude the possibility that after stimulation, certain cells bear these receptors but that the receptors are reduced in number or have decreased binding affinities.

An interesting observation that indicates certain antigens known to elicit a DTH response stimulate NK-cells *in vivo* is that when viable BCG bacilli were injected into the peritoneum of mice, the resultant peritoneal exudate cells displayed high levels of NK activity (20). Our observation that the level of NK-like CMC stimulated in the PBL of a given individual by Candida antigen correlated closely with that individual's sensitivity to that antigen on skin testing, indicates that NK-like CMC may be involved in the DTH response in man.

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