Brief Definitive Report

SPECIFIC LYSIS OF ALLOGENEIC CELLS AFTER ACTIVATION OF CD3⁻ LYMPHOCYTES IN MIXED LYMPHOCYTE CULTURE

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B and T lymphocytes specifically recognize antigens by means of surface receptors (surface immunoglobulins [sIg] and TCR [1], respectively) that represent the molecular product of multiple rearranging genes (2). Other lymphoid cells that lack both sIg and TCR, are generally thought to be unable to recognize antigen(s), but express "nonspecific" cytolytic activity against different types of tumor target cells (3). An important function of $CD3^+$ T lymphocytes is the ability to recognize foreign MHC molecules expressed by allogeneic cells. For example, in mixed lymphocyte cultures (MLC), $CD3^+$ lymphocytes proliferate in response to allogeneic cells and acquire the ability to specifically lyse target cells bearing the stimulating alloantigens (4–6). These events depend not only on the ability to specifically recognize foreign MHC molecules, but also on the production of growth factors allowing proliferation of cytolytic precursors (CTL-P). In this paper we show that TCR/$CD3^-$ peripheral blood cell populations acquire the ability to lyse specific allogeneic target cells after activation in MLC, in the presence of exogenous IL-2. Cloned $CD3^-$ cells displaying a selective cytolytic pattern did not express detectable surface TCR-α/β or γ/δ molecules and lacked productive mRNA for TCR α and β chains. Small amounts of TCR-γ mRNA were detectable in one of four clones tested. mRNA for CD3 γ and δ chains were undetectable in all clones, whereas CD3 ε mRNA was consistently present (7).

Materials and Methods

Isolation of $CD3^-4^-8^-$ Lymphocytes, MLC, Cell Cloning, and Evaluation of Cytolytic Activity. Peripheral blood lymphocytes derived from normal donors were isolated by Ficoll-Hypaque (F-H) gradients and cells were then incubated with a mixture of anti-CD3 (OKT3, Ortho Pharmaceuticals, Raritan, NJ), anti-CD4 (HP26), and anti-CD8 (B9.4) mAbs followed by treatment with rabbit complement for 1 h at 37°C. Viable cells were isolated by F-H gradients and cultured in microwells (5×10^4 cells/well) in the presence of autologous or al-

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logeneic irradiated feeder cells; after 4 d the cultures were supplemented with 100 U/ml of rIL-2. The MLC-derived cells were cloned under limiting dilution conditions in the presence of allogeneic irradiated feeder cells and 100 U/ml of rIL-2. 10 d later the cytolytic activity was tested in a 4-h ^{51}Cr-release assay, in which varying numbers of effector cells were tested against one or another of the following target cells: NK-sensitive K562 cells, PHA blasts derived from the same source of lymphocytes used as stimulating cells in MLC, or from autologous or unrelated lymphocytes. PHA blasts were obtained by culturing PBL for 4 d with 0.5% PHA (vol/vol) in the presence of rIL-2 (100 U/ml). In all instances, target cells were used at 5×10^3/well, for a final E/T cell ratio ranging between 25:1 and 1.5:1. Percent of specific lysis was determined as previously described (6).

Flow Cytofluorometric Analysis. 10^5 cells were stained with the appropriate mAb followed by fluoresceinated goat anti-mouse Ig. Control aliquots were stained with the fluorescent reagent alone. All samples were then analyzed on a flow cytometer (FACS II, Becton Dickinson & Co., Mountain View, CA) gated to exclude nonviable cells. Results are expressed as arbitrary normalized fluorescence histograms, i.e., number of cells vs. fluorescence intensity. The mAbs used in these studies were represented by anti-Leu-4 (anti-CD3, Becton Dickinson & Co.), WT31 (directed to the TCR complex expressed by TCR-α/β-bearing cells; Sambio, Uden, The Netherlands), BB3 (directed to the disulphide-linked form of TCR-γ/δ) (8), δ-TCS-1 (directed to the δ chain of non-disulphide-linked TCR-γ/δ) (9), and MAR 206 (anti-CD2).

Northern Blot Analysis. Total cellular RNA was extracted from frozen cell pellets by the guanidine thiocyanate method and purified by centrifugation through a CsCl gradient. RNA was size fractionated by electrophoresis in a 1.5% agarose gel containing formaldehyde and was transferred by capillary suction to a charged Nylon membrane in 1.5 M NaCl, 0.15 M sodium citrate. Hybridization of the filters with ^{32}P-labeled DNA probes and the washing of the filters were performed as previously described (7). The DNA probes were a 1.1-kb Eco RI fragment of the TCR-α cDNA clone pY14, a 0.77-kb Pst I fragment of the TCR-β cDNA clone JUR-β2, a 1.6-kb Eco RI fragment of the TCR-γ cDNA clone HGPO2, a 0.7-kb Eco RI fragment of the CD3-γ cDNA clone pJ6T3γ-2, two 30-mer oligonucleotides overlapping by 10 complementary bases corresponding to a CD3 δ cDNA (GAGGGAACGGTGGGAACACTGCTCTCAGGAC and CCCAGGTCCAGTCTTGTAATGTCTGAGAGC) and two 30-mer oligonucleotides overlapping by 10 complementary bases corresponding to a CD3 ε cDNA (CCCAGAGGAAGCAAACCAGAAGATGCGAAC and GCCCTCAGGTAGAGATAAAAGTTCGCATCT). Exposure times were 2 d at −70 C.

Results and Discussions

Cell populations enriched in CD3$^-$ lymphocytes were obtained by treatment of nonadherent PBL with mAbs directed to CD3, CD4, and CD8 molecules. The resulting populations were CD3$^-$, CD4$^-$, and CD8$^-$, expressed CD2 (~70%), CD7 (80%), and Leu19 (~75%) and contained ~20% of sIg$^+$ and B1$^+$ cells (as detected by indirect immunofluorescence and microscopical examination).

Cells were cultured in the presence of γ-irradiated (5,000 rad) allogeneic mononuclear cells. At day 4, in a group of cultures, 100 U/ml rIL-2 were added and cultures continued for additional 8–12 d. The resulting cells were tested for their ability to lyse ^{51}Cr-labeled PHA-induced blasts derived from the allogeneic stimulating cells or from autologous or unrelated allogeneic cells. Fig. 1 shows that specific allogeneic cells were efficiently lysed, whereas no activity could be detected against the other target cells, even at high lymphocyte/target ratios. Although not shown, cell proliferation and generation of cytolytic activity were strictly dependent on the addition of exogenous IL-2. The observed cytolytic activity could be explained by the presence of (alloreactive) CD3$^+$ cells contaminating the original responding lymphocyte populations and progressively outgrowing CD3$^-$ cells during

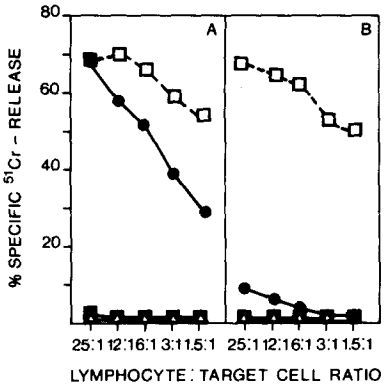

FIGURE 1. MLC-activated CD3$^-$ lymphocytes specifically lyse allogeneic target cells. Cells were stimulated in MLC and tested for cytolytic activity against different ^{51}Cr-labeled PHA-induced blasts or K562 target cells. (*A*) CD3$^-$ cells that had been cultured with the allogeneic cells "a" lysed only target cells "a" (●), but not unrelated allogeneic target cells "b" (■) or autologous cells (Δ). (*B*) The same cell population, after culture in the presence of autologous lymphocytes, was unable to lyse autologous or allogeneic target cells. On the other hand, lysis of K562 occurred independent on the autologous or allogeneic stimulus provided (□).

culture. This possibility was ruled out by the demonstration that the cytolytic populations neither reacted with a panel of anti-CD3 mAbs (Leu4, OKT3, UCHT1, JT3) nor bound mAbs specific for different types of TCR (WT31, δ-TCS-1 or BB3 mAbs) (8–9), as detected by FACS analysis (Fig. 2 *A*). Thus, the surface phenotype of these MLC-derived cells was the following: CD7$^+$CD2$^+$CD3$^-$sIg$^-$B1$^-$. Parallel control cultures of CD3$^-$ cells cultured (in IL-2) in the absence of stimulating cells or in the presence of autologous irradiated cells, proliferated considerably less (not shown) and, more importantly, did not result in generation of cytolytic activity either against autologous or allogeneic target cells (Fig. 1). Therefore, the generation of specific cytolytic activity against allogeneic cells depends not only on the availability of IL-2, but also on the delivery of a specific stimulus provided by allogeneic cells. On the other hand, comparable levels of cytolytic activity against K562 cells were detected

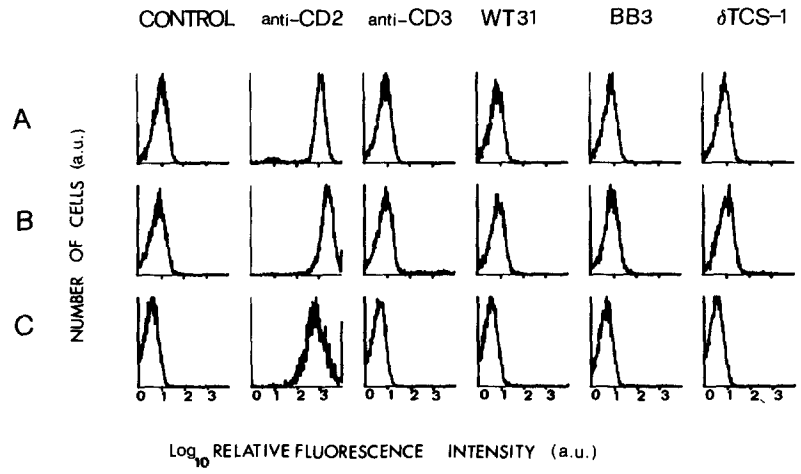

FIGURE 2. Surface antigens expressed by a MLC-activated CD3$^-$ lymphocyte population (*A*) and two representative clones with specific cytolytic activity against allogeneic PHA-blasts. (*B*) Clone G10; (*C*) clone H12.

in MLC-derived $CD3^-$ cells regardless of the origin (autologous or allogeneic) of the stimulating cell population (Fig. 1).

We next analyzed clones derived from MLC-stimulated $CD3^-$ cells. These clones were screened on the basis of their ability to lyse specific allogeneic target cells (used as stimulator in MLC). Table I shows a group of representative clones obtained from four different individuals. It is evident that all such clones expressed neither CD3 nor TCR molecules at the cell surface (Fig. 2, *B* and *C*), nevertheless, they lysed specific target cells but not unrelated allogeneic or autologous blast cells. K562 target cells were highly susceptible to lysis, whereas NK-resistant fresh melanoma cells displayed variable susceptibility to lysis. A further evidence that lysis of specific target cells was not mediated by conventional CD3/TCR molecules was provided by the finding that mAbs directed to CD3 molecules failed to inhibit (or enhance) the specific target cell lysis; on the other hand, the same mAbs blocked the specific target cell lysis mediated by conventional CTLs (not shown). The failure of anti-CD3 or anti-TCR mAbs to bind to the cells examined does not rule out the possibility that these molecules may be expressed at the cell surface. Indeed they may be expressed in a molecular configuration that would not be recognized by conventional anti-CD3 mAbs. We therefore examined whether mRNA for different chains of CD3 or TCR-α, -β, and -γ chains could be detected in cloned alloreactive $CD3^-$ cells. No mRNA for TCR-α and a truncated (1 kb) form of TCR β chains were detected in the four clones analyzed (two representative clones are shown in Fig. 3). mRNA for the TCR-γ chain was absent in three clones and detectable in small amounts in one (Fig. 3). No mRNA for CD3 γ and δ chains could be detected, whereas high levels of mRNA for the ε chain were consistently present (7). These data further support the concept that no conventional CD3 or TCR molecules are surface expressed in $CD3^-$ cells capable of specific lysis of allogeneic cells.

TABLE I

Cytolytic Activity of Allogeneic MLC-derived $CD2^+$ $CD3^-$ Clones

	^{51}Cr-labeled target cells				
Clone	Autologous PHA-blasts	Specific allogeneic PHA-blasts	Unrelated allogeneic PHA-blasts	K562	Fresh melanoma cells
E6	1*	86	1	73	25
E13	0	43	0	74	13
E19	0	37	0	74	8
E35	5	75	ND	53	11
E26	1	80	1	ND	ND
E5	0	57	ND	81	ND
C8	0	100	0	ND	35
H12	0	85	0	51	26
G10	0	48	0	89	31
D5	0	63	0	78	4
E3	0	62	0	100	100
E4	0	100	2	100	86
E29	0	57	4	100	48

* Results are expressed as percent of specific ^{51}Cr release at a lymphocyte/target cell ratio of ~3:1.

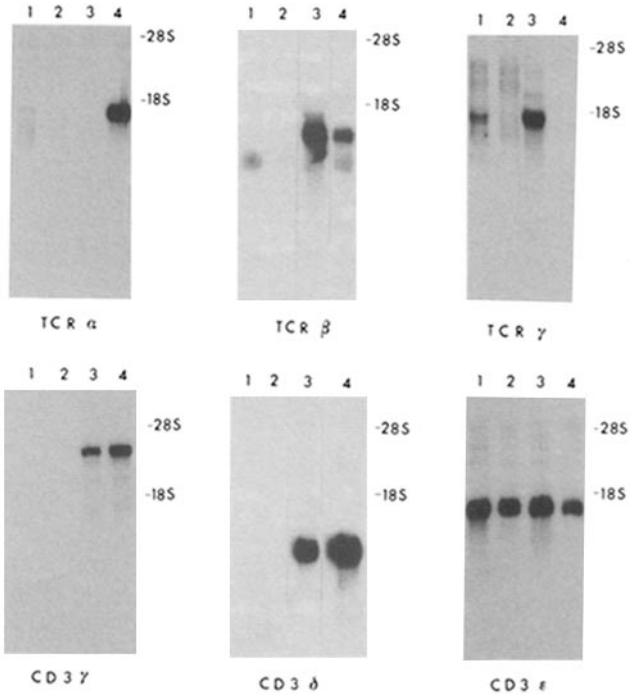

FIGURE 3. Northern blot analysis of TCR and CD3 gene products in two representative $CD3^-$ clones with specific cytolytic activity against allogeneic blasts bearing the stimulating alloantigens. (Lane *1*) clone H12; (lane *2*) clone G10; (lane *3*) Peer leukemic cell line; and (lane *4*) Jurkat leukemia. The blots were hybridized to the TCR-α, -β, -γ, and to CD3 γ, δ, and ε probes.

While the ability to generate $CD3^-$ lymphocytes capable of specific lysis of allogeneic cells has been repeatedly demonstrated in all four normal donors tested, not all individuals were equally able to act as stimulators. Thus, only 2 of 10 stimulators efficiently induced $CD3^-$ cell proliferation and cytolytic activity.

The molecular basis of specific target cell lysis mediated by $CD3^-$ cells remains obscure at the present. Our data are compatible with the hypothesis of the presence of surface receptor molecules, perhaps displaying a limited variability, capable of recognizing allelic determinants (non-MHC-encoded?) expressed by allogeneic cells. Whatever the explanation would be, the finding that (at least certain) $CD3^-$ lymphocytes can specifically recognize and lyse allogeneic cells may have important implications in transplantation and the possibility of "in vivo" responses mediated by $CD3^-$ lymphocytes should be carefully explored.

Summary

Human $CD3^-$ lymphocyte populations were obtained by treating peripheral blood lymphocytes with mAbs directed to CD3, CD4, and CD8 surface antigens. The resulting populations were cultured with irradiated allogeneic cells; at day 4, 100 U/ml IL-2 were added and cultures continued for an additional 10 d. The resulting populations were $CD3^-CD2^+CD7^+$ and displayed cytolytic activity against PHA-induced blast cells bearing the stimulating alloantigens but not against autologous or unrelated allogeneic blast cells. When $CD3^-$ populations were cultured with irradiated autologous cells, no cytolytic activity could be detected either against autologous or allogeneic blast cells. On the other hand, K562 target cells were lysed

by both MLC-derived $CD3^-$ cell populations regardless of the origin (autologous or allogeneic) of the stimulating cells. $CD3^-$ clones were further derived from MLC-stimulated $CD3^-$ populations. These clones displayed a cytolytic pattern similar to the original MLC populations as only specific PHA blasts could be lysed. These clones did not express detectable surface TCR-α/β or -γ/δ molecules and lacked productive mRNA for TCR α and β chains, while small amounts of TCR-γ mRNA were detectable in one of four clones tested. Also mRNA for CD3 γ and δ chains were undetectable in all clones, however, CD3 ε mRNA was consistently present.

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