Brief Definitive Report

SELECTIVE INHIBITION OF HUMAN T CELL CYTOTOXICITY AT LEVELS OF TARGET RECOGNITION OR INITIATION OF LYSIS BY MONOCLONAL OKT3 AND Leu-2a ANTIBODIES*

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Monoclonal antibodies directed against surface antigens expressed on subpopulations of lymphoid cells might be used for selective interference with function mediated by these structures. Several anti-human T cell monoclonals have been shown to interfere with immune responses when present in an assay. A class of 33,000 M_r antigens expressed on cytotoxic/suppressive cells are detected by OKT5, OKT8, and OKT8a antibodies. Addition of these antibodies to a lytic assay reduces lysis (1, 2). Anti-Leu-2a, binding to the same population of cells, precipitates molecules of 32,000 and 43,000 M_r (3). In the presence of this antibody, the mixed leukocyte reaction (MLR) is diminished, and cytotoxic T lymphocyte (CTL) activity is prevented (4, 5). Another cell surface antigen detected by OKT3 antibodies is present on all peripheral T cells and has an M_r of 19,000 (2). Interference with this antigen by the antibody gives rise to a mitogenic response, but in higher concentrations the induction of T cell responses as well as the execution of T killing is blocked (6, 7). In this study, we approached the question of what mechanisms are interfered with by the presence of such antibodies in a lytic assay. Out of a panel of seven anti-human lymphoid cell monoclonals, three were shown to diminish CTL activity, whereas none affected natural killer cell (NK) activity. OKT3 and Leu-2a antibodies were investigated further. In both cases, the inhibition resulted from an interaction with the effector cells. The inhibition induced by Leu-2a antibodies was rapidly reversible, whereas anti-OKT3 had a more long-lasting influence. With the aid of a single cell assay, anti-Leu-2a was shown to block a target-binding step, whereas anti-OKT3 allowed such binding but inhibited the subsequent lytic step.

Materials and Methods

Cell Culture. Peripheral blood mononuclear cells were prepared from the blood of healthy volunteers by Ficoll-Hypaque floatation. MLR were established at 1×10^6 responders and 1×10^6 2,500 rad irradiated stimulators per ml. Concanavalin A (Con A) stimulation of the cells to be used as targets was performed at 5×10^5 cells/ml using 2.5 μg/ml of Con A. *Staphylococcus aureus* stimulation was effected at 1×10^6 cells/ml with 0.1% (vol/vol) formalin-fixed bacteria (8). The stimulated leukocytes were depleted of sheep erythrocyte-rosetting cells (9) before being used as B cell targets. As an NK-sensitive target, MOLT-4 was used (10). Cells were grown in RPMI 1640, supplemented with 2 mM glutamine, 50 μM 2-mercaptoethanol, and 50

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IU penicillin and 60 μg streptomycin per ml. The medium contained 5% responder plasma with 10 IU heparin per ml in the lymphocyte stimulations, and 5% heat-inactivated fetal calf serum in the tumor cultures and lytic and binding assays.

Monoclonal Antibodies. Anti-Leu-1, anti-OKT3, and anti-OKT-11 detect all human T cells (11, 6, 12); anti-Leu-2a and anti-OKT8 bind to the cytotoxic/suppressive T subset (3, 1); anti-Leu-3a bind to helper T cells (3); and anti-OKM 1 has affinity for monocytes (13). The OK series of antibodies were donated by Ortho Pharmaceutical, Raritan, NJ. The Leu antibodies were provided by Becton, Dickinson & Co., Sunnyvale, CA. Effector cells were preincubated with the antibodies for 30 min at 37°C before being mixed with target cells.

Chromium Release Assay. Target cells were labeled with ^{51}chromium and added to dilutions of effector cells made in duplicate or triplicate in V-bottomed microtiter plates. After a 4-h incubation at 37°C, the plates were spun, and aliquots of supernatant were collected for determination of radioactivity. Specific release was calculated as ([experimental release − spontaneous release]/[maximum release − spontaneous release]) × 100%.

Single Cell Binding and Cytotoxicity Assay. Binding of target cells to effectors and their subsequent lysis was investigated according to Grimm et al. (14), modified as pointed out below. In brief, effector cells were preincubated in 10 μg/ml anti-OKT3, 5 μg/ml anti-Leu-2a, or medium. They were not washed before the assay. Target cells were labeled with carboxy fluorescein diacetate (15) to allow their distinction from effectors under a fluorescence microscope. Equal volumes of effector and target cells were mixed at a one to one ratio and spun together at 500 *g*. After 10 min incubation at 37°C, the pellet was resuspended and the cells mixed with the agarose solution and then dripped onto petri dishes. Parallel dishes were either scored for binding immediately or incubated with medium for 3 h and then stained with trypan blue and fixed. The percentage target binding cells (TBC) was calculated after scoring 400 effector cells. The fraction of conjugates containing dead cells after the 3-h incubation was expressed as the percentage cytotoxic target binding cells (CTBC), estimated by counting 100 conjugates.

Results

Three Monoclonal Antibodies Inhibit T Cell Killing without Affecting NK Activity. A panel of monoclonal antibodies was added at 2 μg/ml for a 30-min preincubation of MLR cells. These were then assayed for lytic activity against Con A blasts established from the stimulator and against an NK-sensitive tumor cell line, MOLT-4. The antibody concentration at the highest effector to target ratio investigated was 1 μg/ml. Fig. 1 gives the mean reduction in lytic activity by the effectors. Reduced CTL activity was seen after treatment with OKT3, OKT8, and Leu-2a antibodies ($P < 0.001$). No monoclonals affected NK activity. Control experiments using *S. aureus*-stimulated leukocytes depleted of T cells clarified that the inhibition resulted from an interaction with the effector cells for anti-OKT3 and Leu-2a, the two antibodies chosen for a more detailed study (data not shown). This was previously shown for anti-OKT3 by Platsoucas and Good (2).

Inhibition by Leu-2a Antibodies Is Rapidly Reversible. The anti-OKT3-induced inhibition of lysis was shown to be proportionally increased by applying the same amount of antibody in a smaller volume during preincubation. For anti-Leu-2a, the extent of the inhibition was set by the concentration of antibody in the test (Fig. 2). This indication of differential reversibility of the inhibition exerted by OKT3 and Leu-2a antibodies was confirmed by washing the effectors after the antibody treatment. With this protocol, anti-Leu-2a failed to affect lysis, whereas for anti-OKT3, inhibition was not affected. After resting the OKT3-treated and subsequently washed cells for 3 h at 37°C, inhibition was reduced by 50%, and control levels of lysis were usually obtained by 6 h. The faster reversibility for the anti-Leu-2a inhibition of CTL activity might

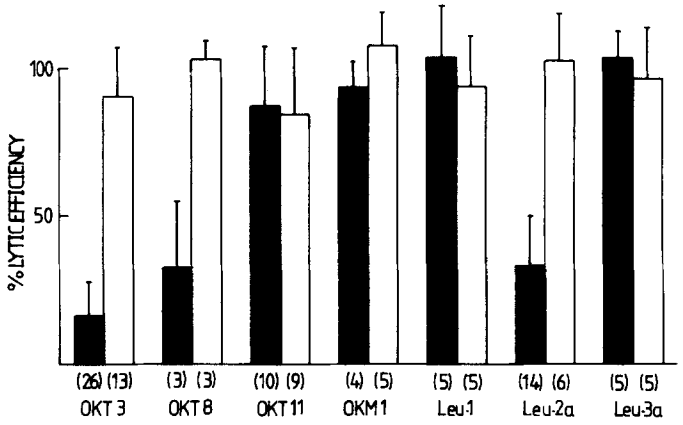

FIG. 1. The fraction of lytic units remaining in populations of MLR effectors after treatment with the indicated antibodies are represented as percent of control ± SD. Lysis was measured for the relevant T cell targets (filled bars) and for MOLT-4 cells (open bars). The numbers of observations are given in parentheses.

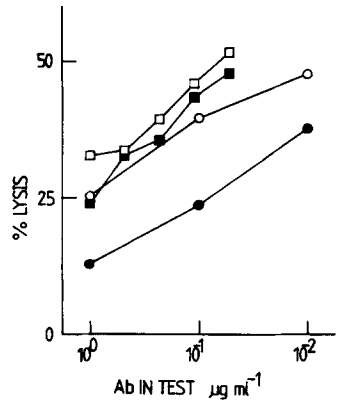

FIG. 2. MLR cells were treated in graded amounts of OKT3 (○,●) or Leu-2a (□,■) antibodies. Cell concentration during antibody treatment was two times (○,□) or 20 times (●,■) that in the assay. The antibody concentrations, after diluting the preincubated effectors and mixing with target cells, are indicated on the abscissa. The ratio of targets to effectors was 50:1. Percent lysis in the absence of antibody was 52.9%.

reflect a faster turnover rate for the corresponding surface antigen or a lower avidity of the antibody.

OKT3 and Leu-2a Antibodies Prevent Target Lysis by Different Mechanisms. To analyze at what step the lytic process is inhibited by these antibodies, we applied a single cell assay in agarose. In Table I, results are presented from experiments where anti-OKT3 was added to effectors before introducing them to their target cells. Decreased target lysis was evident from chromium release data. The number of effector cells that bound targets was not detectably influenced. Bound targets, however, survived the interaction more often when the effectors had been treated with anti-OKT3. The case for anti-Leu-2a was investigated in another set of MLR (Table II). With a similar overall effect upon lysis, these antibodies could be shown to substantially reduce target binding. The cytotoxicity was determined in a standard chromium release assay at an effector to target ratio of 20:1. In the binding test a ratio of 1:1 was used. This difference, together with the prevented recycling of CTL in the agarose test, contributes to the lack of direct correspondence between the percent cytotoxicity and numbers obtained in the agarose test.

TABLE I

OKT3 Monoclonal Antibodies Prevent Cytotoxicity by Reducing Lytic Activity without Affecting the Target-Binding Ability

MLR	Antibody	Target	Cytotoxicity	TBC	CTBC
			%	%	%
A anti-B	0	B	35.4	8.5	60
A anti-B	OKT3	B	8.1	8.0	27
A anti-B	0	A	ND*	1.5	0
A anti-C	0	C	37.4	6.8	71
A anti-C	OKT3	C	9.9	5.8	24
A anti-C	0	A	ND	1.4	0
D anti-E	0	E	44.1	10.0	36
D anti-E	OKT3	E	7.5	9.6	14
D anti-E	0	D	−7.8	ND	ND

Three MLR were investigated for lytic activity in the presence or absence of OKT3 antibodies. Cytotoxicity was measured in a chromium release assay. The percentage TBC was determined using a single cell assay. The fraction of conjugates containing killed cells after a 3 h incubation is represented as CTBC.

* Not done.

TABLE II

Leu-2a Monoclonal Antibodies Prevent Cytotoxicity by Inhibiting Target

MLR	Antibody	Target	Cytotoxicity	TBC	CTBC
			%	%	%
F anti-G	0	G	33.7	9.9	40
F anti-G	Leu-2a	G	3.9	4.5	38
F anti-H	0	H	58.5	6.4	35
F anti-H	Leu-2a	H	4.6	0.0	0
I anti-J	0	J	36.7	10.9	36
I anti-J	Leu-2a	J	7.6	3.4	34

The impact of Leu-2a antibodies on target binding and lysis was measured, similar to that presented in Table I.

Discussion

A number of immune reactions are modified by the presence of monoclonal antibodies with affinity for participating cells. In the work presented here, we directed our attention to the effects of some T-specific antibodies on a short-term immune reaction, the effector phase of cell mediated lysis. In a panel of six anti-human T cell antibodies and one directed at the monocyte series anti-OKT3, binding to all T cells and anti-Leu-2a and anti-OKT8, detecting the cytotoxic/suppressive T cell subset, were shown to prevent CTL but not NK activity. This selectivity makes these antibodies useful for assigning a particular lytic activity to one or the other effector classes. Leu-2a and OKT3 antibodies were investigated in greater detail. Both were shown to prevent CTL activity by an interaction with the effector cell, using non-T target cells. A difference for the two antibodies was noted in that Leu-2a antibodies required being present in the assay to exert their full inhibition, whereas washing away free OKT3 antibody before the assay did not influence inhibition. This allows for treatment with this latter antibody of large numbers of cells in a small volume before adding them to the test. OKT3 antibody concentrations from 20 ng/ml reproducibly induced detectable inhibition.

The lytic process by CTL cells has been separated in several distinct steps (16). Inhibition of lysis could result from interference with several of these steps. A single cell assay contributes important information. With this technique, the reduced lysis observed after anti-OKT3 treatment was shown not to result from reduced target

binding, but rather the ability to lyse bound cells was impeded. The Leu-2a antibody, in contrast, caused a diminished binding of target cells by antibody-treated effectors.

The ability of the Leu-2a antibody to affect the target binding step of CTL activity as well as the rapid reversibility of the effects of antibody treatment accord with what has been shown for antibodies to the Lyt-2 antigen in the mouse (17, 18). A homology between these two antigens has been proposed because of similarities in structure and occurrence together with the effects upon lymphocyte stimulation and execution of lytic activity by antibodies to these antigens (3). The mouse Lyt-2 antigen has been shown to be coded for by a gene segment closely linked to that for the kappa chain (19). The expression of the Lyt-2 antigen has been suggested to primarily correlate with the class of major histocompatibility complex antigen perceived by the cell rather than with it's function (20). Together with the impaired target binding by CTL cells in the presence of Lyt-2 antibodies, these observations suggest a relatedness of this antigen to the antigen recognition structure on cytotoxic T cells. The results presented here strengthen the analogy between the human Leu-2a antigen and the Lyt-2 antigen in the mouse and in consequence point to Leu-2a as an antigen potentially forming part of a human antigen recognition structure on T cells. Such a role has previously been suggested for the OKT3 antigen (7). The failure of OKT3 antibodies to detectably affect target binding in doses that severely depress target lysis makes this concept less tenable. The intriguing effects of the OKT3 antibody, including low dose mitogenicity (7), and in higher concentrations, blocked proliferative responses by T cells (6) and inhibited CTL activity via a mechanism distinct from target recognition (this paper), however, indicate a crucial function of this antigen. Taken together, these two antibodies, here subjected to analysis, by their different ways of affecting cell function constitute attractive tools for the investigation of T lymphocyte activity in general and specifically as it applies to the lytic phase of a response.

Summary

Out of a panel of seven monoclonal antibodies with affinity for human lymphoid cells, three were shown to prevent cytotoxic T cell activity, whereas none affected natural killer cell activity when applied without complement. Anti-OKT3 and anti-Leu-2a, with affinity for all T cells and the cytotoxic/suppressive subset, respectively, were both shown to inhibit T killing by their interaction with the effector cell. For anti-OKT3, the inhibition remained after free antibody was washed away. Anti-Leu-2a, in contrast, induced a rapidly reversible inhibition. Using a single cell assay, anti-OKT3 was shown to reduce the lytic ability without affecting target cell binding, whereas anti-Leu-2a prevented the effectors from binding target cells.

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