COOPERATIVE INTERACTION OF FACTOR B AND OTHER COMPLEMENT COMPONENTS WITH MONONUCLEAR CELLS IN THE ANTIBODY-INDEPENDENT LYSIS OF XENOGENEIC ERYTHROCYTES*

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We previously reported (1, 2) that normally noncytotoxic mononuclear cells from human peripheral blood and a heat-labile, nonantibody factor(s) present in normal human serum can cooperate in vitro to lyse a variety of xenogeneic erythrocyte target cells. Because neither serum nor the mononuclear cells alone cause significant target lysis, we termed this cytotoxic system synergistic cytotoxicity. In our earlier studies, we showed that the serum factor active in this system was labile to heating (56°C, 30 min) and to treatment with zymosan and cobra venom factor, required divalent cations, and was present in agammaglobulinemic sera and sera depleted of immunoglobulins by affinity chromatography. Both monocytes and T lymphocytes were found to kill xenogeneic erythrocytes in the presence of these active sera. One particularly intriguing finding of these earlier studies was the observation that antibodies to factor B of the alternative complement pathway, when added to the active normal serum, substantially inhibited target cell lysis in this system. These observations suggested a previously unrecognized link between elements of two major nonimmune host defense systems, the alternative complement pathway and potentially cytotoxic mononuclear cells.

This report presents studies further characterizing the serum factors active in this system by using sera deficient in, or depleted of, single complement components and/or highly purified individual complement components tested with purified lymphocytes, monocytes, or mixtures of the two cell types.

Materials and Methods

Media. RPMI 1640 and Hanks' balanced salt solution were purchased from Microbiological Associates, Bethesda MD. Hepes buffer was from Gibco Laboratories, Grand Island Biological Co., Grand Island, NY.

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Serum Samples. As previously described (1), both heparinized human plasma (20 U/ml) and serum from normal individuals support synergistic cytotoxicity equally well. Human sera deficient in C3, C5, C7, and C8 were obtained from genetically deficient individuals and have been previously described (3–7). C4-deficient guinea pig serum was obtained from genetically deficient guinea pigs (8). Normal human serum was depleted of factor B using solid-phase $F(ab')_2$ anti-factor B immunoabsorbent columns as described (15). Factor D-depleted serum was prepared by gel filtration (9). All sera or plasmas were absorbed four times against 1/3 volume of packed target erythrocytes on ice, as previously described, to remove any target cell-specific antibody (1). All serum and plasma sources were tested for residual antibody contamination by the ability of heat treatment (56°C, 30 min) to abolish lysis of target cells by human mononuclear cells in the absorbed plasma. Such treatment does not affect antibody-dependent cellular cytotoxicity (ADCC) (1). By these criteria, all of the serum and plasma sources were totally depleted of target-specific antibody.

Sources of Antibodies. Goat anti-C3 and anti-C5 antisera were purchased from Miles Laboratories Inc., Elkhardt, IN. Goat anti-factor B antiserum was purchased from Atlantic Antibodies, Scarborough, ME. A high titer mouse monoclonal hybridoma antibody directed against C3 purified from tissue culture supernatants was kindly provided by Dr. J. D. Capra, University of Texas Health Science Center, Dallas, TX. 1 mg of this antibody inactivates >1 mg of C3 detected by hemolytic assay (14). All antibodies were absorbed against chicken erythrocytes and were heat-inactivated (56°C, 30 min) before use.

Cytotoxicity Assay. Cytotoxicity was performed using ^{51}Cr-labeled chicken erythrocyte targets in an 18-h ^{51}Cr-release assay run at a target-to-effector cell ratio of 1:10, as described (1, 2). When serum or plasma were tested for ability to support synergistic cytotoxicity, 25 μl of a 1:5 dilution in medium was added to each microtiter well (total volume, 200 μl/well). Some experiments contained added antiserum (25 μl of 1:5 dilution) or monoclonal anti-C3 (25 μl of solution containing 140 μg antibody/ml). In these experiments, wells with human serum, antiserum or monoclonal antibody, and effector leukocytes were allowed to incubate 30 min at 37°C before adding targets.

Assays were performed in triplicate in serum-free RPMI 1640 with added penicillin (50 U/ml), streptomycin (50 μg/ml), and glutamine (4 mM) and were harvested on a Titertek supernatant collection system (Flow Laboratories, Bethesda, MD). Results are expressed as mean percent ^{51}Cr release compared with total detergent-releasible counts. All assays were performed in triplicate, with ≤5% variability.

Purified Factor B and C5. Purified human factor B was prepared from normal human serum as previously described by Kerr and Porter (16). Factor B was diluted in medium to a concentration approximately that of normal serum (titer 40,000) and used in 6–25 μl aliquots per microtiter well. Most factor B preparations exhibited single bands on sodium dodecyl sulfate-polyacrylamide gel electropheresis; some preparations had an additional faint band consistent with the major cleavage product of factor B (Bb). C5 was prepared from normal human serum, according to published techniques (17), and further passed over immunoabsorbent columns of anti-C3, anti-C6, and Staphylococcal protein A. C5 preparations were free of other detectable complement components by hemolytic assay (14).

Preparation of Cells. Whole mononuclear cells were prepared by buoyant density centrifugation using lymphocyte separation medium (Litton Bionetics, Kensington, MD) (1). Monocyte-enriched populations were obtained from whole mononuclear cell preparations by adherence to plastic petri dishes in RPMI 1640 containing 10% heat-inactivated fetal bovine serum with added Hepes buffer (10 mM), penicillin, streptomycin, and glutamine. Monocytes were allowed to adhere for 60 min at 37°C. The nonadherent cells (lymphocytes) were gently washed off with warm phosphate-buffered saline, and the residual cells were then incubated with a buffered EDTA solution (Versene; Gibco Laboratories, Grand Island Biological Co.) for 20 min, followed by vigorous aspiration with a pasteur pipet. All cells were washed two additional times in Hanks' balanced salt solution. Monocyte-enriched preparations contained from 75–95% alpha naphthyl butyrate esterase-positive cells (10). The nonadherent lymphocyte population contained <5% esterase-positive cells.

The RPMI 1056 human B cell line was kindly supplied by Dr. Stanley Korsmeyer, National Institutes of Health. The line was grown in RPMI 1640 medium containing 10% fetal calf

serum, penicillin, streptomycin, and glutamine. Cells were collected at 300 *g* for 10 min and washed three times with Hanks' balanced salt solution before use.

Results

C5-deficient and Factor B-depleted Sera Do Not Support Synergistic Cytotoxicity by Whole Mononuclear Cells. To further characterize the serum requirements for the expression of synergistic cytotoxicity, we performed a series of experiments that examined the ability of human sera deficient in single complement components to support cytotoxicity in the presence of human mononuclear leukocytes. Table I presents a summary of these experiments. As can be seen, of the genetically deficient sera tested, only serum deficient in C5 failed to support some target cell lysis by mononuclear effector cells. By marked contrast, normal serum and serum deficient in C3 cooperated with mononuclear cells in causing target cell lysis. Sera deficient in C4, C7, and C8 all supported some target cell lysis, but usually less than normal serum. It should be noted, however, that the range of activity found in sera from various normal subjects is broad in this assay. In 30 assays on 11 different normal sera, mean percent ^{51}Cr release was 55.2, with a standard deviation of 23.9. Also of note is that some of the deficient sera had been stored at −90°C for periods in excess of 1 yr, which could potentially affect the activity.

Table I also includes the results of studies of sera depleted of factor B (by affinity chromatography) and of factor D (by gel filtration). Factor D-depleted serum supported moderate levels of target lysis, whereas serum depleted of factor B was totally inactive in this lytic system. As shown in our earlier studies (1, 2), antibody to factor B was markedly inhibitory to target cell lysis. Table II demonstrates that antibodies to C5 also inhibit target cell lysis in this cytotoxic system. Thus, these experiments indicate that both C5 and factor B appear to be essential serum components for target cell lysis by unfractionated mononuclear effector cell populations.

TABLE I

Synergistic Cytotoxicity by Whole Mononuclear Cell Effectors in the Presence of Complement-deficient Sera

Serum	Percent ^{51}Cr-specific release	Number of experiments
C3-deficient	66.7 ± 7.4	3
C4-deficient (guinea pig)	47.0 ± 7.5	3
C5-deficient	0.5 ± 0.5	4
C7-deficient	25.3 ± 1.8	3
C8-deficient	37.3 ± 4.1	3
Factor B-depleted	2.0 ± 2.0	5
Factor D-depleted	30.3 ± 17.3	3
Normal	55.2 ± 4.4*	30

Results expressed as mean percent ^{51}Cr release (± 1 SE) of several experiments run in triplicate. Concurrently, normal sera were tested in each experiment. Spontaneous lysis of ^{51}Cr-labeled chicken erythrocytes in the presence of serum-free mononuclear cells has been subtracted from each value (spontaneous lysis ranged from 4–14%; mean, 7.2). Control sera were from 11 normal individuals, whereas deficient sera were from single individuals. Two different preparations of factor B-depleted sera were tested, with similar results in each case.

* SD, 23.8.

TABLE II

Synergistic Cytotoxicity: Effect of Adding Anti-C3 To Normal and C3-deficient Sera

Addition	Percent ^{51}Cr release				
	Experiment 1	Experiment 2	Experiment 3	Experiment 4	Experiment 5
Medium	7	5	12	8	11
N1 plasma*	38	37	65	78	39
N1 plasma + anti-C3 (I)	49	—	—	72	—
N1 plasma + anti-C3 (II)	71	—	—	—	—
N1 plasma + anti-factor B	12	—	—	—	—
N1 plasma + anti-C5	7	—	—	—	—
C3-deficient serum	—	77	78	—	63
C3-deficient serum + anti-C3 (I)	—	68	59	—	—
C3-deficient serum + anti-C3 (II)	—	—	—	—	91
Anti-C3 (I) alone	6	—	12	6	—
Anti-C3 (II) alone	7	—	—	—	7

Five separate experiments showing that anti-C3 fails to abrogate synergistic cytotoxicity. Experiments 1–4 used whole mononuclear cell effectors; purified monocytes were tested in experiment 5. Anti-C3 (I) is a monoclonal mouse antibody, and anti-C3 (II) is a goat antiserum. Note that neither of the anti-C3 antibodies by themselves induced any cytotoxicity.

* N1 plasma (normal plasma) used in all experiments except experiment 1, in which normal serum was used. Normal plasma and serum support synergistic cytotoxicity equally well.

Anti-C3 Antibodies Do Not Inhibit Synergistic Cytotoxicity in the Presence of Normal or C3-deficient Serum. The apparent lack of a requirement for C3 suggested a unique pathway for activity of the serum complement system. Because genetically deficient plasma may still contain small amounts of C3 and because normal monocytes can synthesize C3 (11), we asked whether such small amounts could suffice to support synergistic cytotoxicity. To address this question, antibodies to C3 were added to the C3-deficient serum. Two sources of anti-C3 antibody, one a goat heteroantiserum and one a mouse monoclonal anti-C3, were used. These antibodies were added to cells in the presence of target-absorbed normal human serum or human serum genetically deficient in C3. With both sources of antibody, killing was not inhibited (Table II). In contrast, anti-C5 and anti-factor B markedly inhibited synergistic target cell lysis. 1 mg of the mouse monoclonal anti-C3 was capable of inhibiting >1 mg of hemolytically active C3 (fluid-phase sheep cell assay; data not shown). Because the C3-deficient serum had undetectable immunochemical or hemolytically active C3 (<0.1% of activity in normal serum), the amount of monoclonal anti-C3 used in Table II would be sufficient to inactivate >1,000 times the amount of C3 potentially present in C3-deficient serum used, assuming the C3-deficient serum contained C3 at a concentration just below the limit of detection.

We next performed a series of experiments to determine whether the sera that failed to support synergistic cytotoxicity could be reconstituted. Initially, C5-deficient serum was mixed with an equal volume of factor B-depleted serum. This mixture was fully active in the cytolytic assay (Fig. 1), indicating that the lack of activity in each separate serum was probably not due to the presence of some inhibitory substance. A more direct approach was to reconstitute each deficient serum with the appropriate purified component. As also shown in Fig. 1, each deficient serum regained full

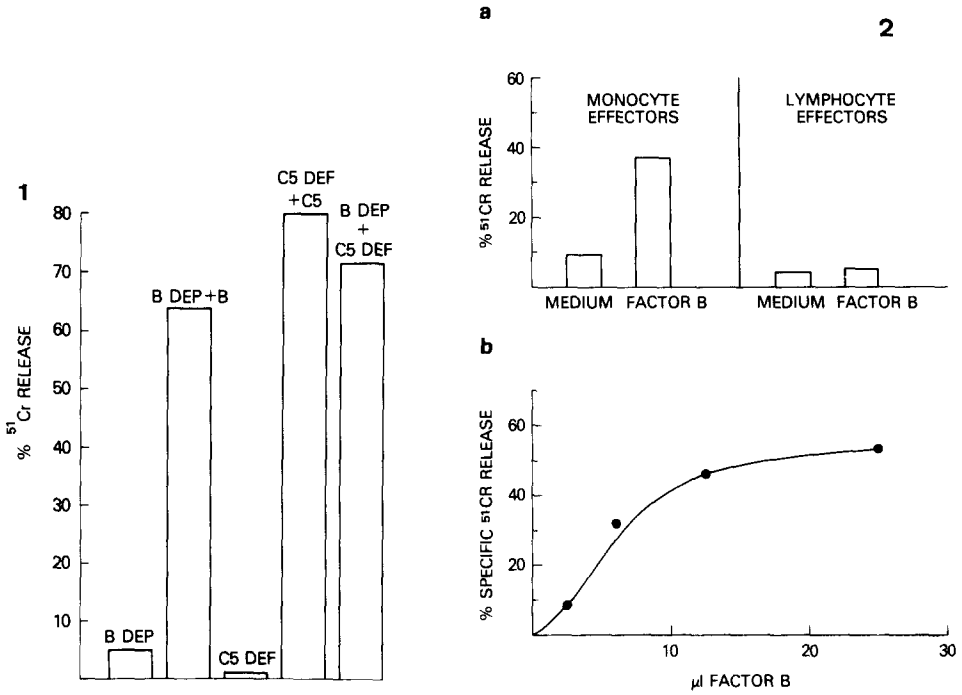

FIG. 1. Whole mononuclear cell effector populations exhibited cytotoxicity when factor B-depleted or C5-deficient sera were reconstituted with the appropriate factors. Results are expressed as percent ^{51}Cr release. Factor B and C5 were added to reconstitute the deficient serum to physiological levels of each component. Representative experiment of four similar studies.

FIG. 2. (a) Cytotoxicity assays were performed in the presence or absence of 25 μl of purified factor B (titer 40,000) in serum-free medium. Final volume is 0.2 ml/microtiter well. Target-to-effector ratio was 1:10 using 3×10^5 enriched monocytes as effectors. (b) Dose-response of factor B-induced monocyte cytotoxicity. Varying amounts of factor B solution (titer 40,000) in medium were added to a microcytotoxicity assay using 3×10^5 enriched monocytes. ^{51}Cr release in the presence of medium alone (12%) was subtracted from each point to yield percent specific ^{51}Cr release.

activity after reconstitution with its missing component.

C5-deficient Serum Supports Synergistic Cytotoxicity by Monocytes but Not by Lymphocytes. Because our previous data had shown that both monocytes and lymphocytes could mediate target cell lysis, we examined the effects of deficient sera on monocyte-enriched and lymphocyte-enriched populations. As can be seen in Table III, factor B-depleted serum failed to support significant killing by either monocytes or lymphocytes. Surprisingly, C5-deficient serum supported monocyte-mediated lysis of target cells but failed to support lymphocyte-dependent target cytolysis (compare with Fig. 1). C7- and C8-deficient sera supported monocyte-mediated cytolysis, albeit less effectively than whole serum, while failing to support lymphocyte-mediated cytolysis. As noted previously, many of these deficient sera had been stored in the freezer for prolonged periods, which potentially could contribute to the depressed activity in these studies. Mixing factor B-depleted serum with either C7-deficient or C8-deficient serum reconstituted full activity of lymphocyte-mediated cytolysis, thus, again making unlikely the possibility of an inhibitor in these deficient sera. Sufficiently purified C7 and C8 were not available to test whether lymphocyte-mediated cytotoxicity may be reconstituted by adding purified components to these deficient sera. Thus, these data

TABLE III

Failure of Factor B-depleted Sera to Support Lysis of Xenogeneic Targets by Separated Monocytes or Lymphocytes

Experiment	Serum or plasma source	Percent specific ^{51}Cr release	
		Monocytes	Lymphocytes
1	Normal	52	54
	Factor B-depleted	8	3
	C7-deficient	23	0
	C8-deficient	38	0
	C7-deficient + B-depleted	—	46
	C8-deficient + B-depleted	—	48
	Factor D-depleted	25	39
2	Normal	91	85
	C3-deficient	60	31
	C5-deficient	22	8

Synergistic cytotoxicity by separated monocytes and lymphocytes showing the results of two representative experiments. The background lysis in the absence of plasma but presence of cells has been subtracted to yield percent specific ^{51}Cr release. For experiment 1, the background release was 8% and 10% ^{51}Cr release for monocytes and lymphocytes, respectively; for experiment 2, the lysis was 9% and 4%, respectively.

TABLE IV

Inhibition of Factor B-induced Monocyte Cytotoxicity by Peripheral Blood Lymphocytes

Addition	Percent ^{51}Cr release		
	Experiment 1	Experiment 2	Experiment 3
Monocytes + medium	8	12	12
Monocytes + factor B	32	61	59
Mononuclear cells + medium	11	—	—
Mononuclear cells + factor B	6	—	—
Monocytes + mononuclear cells + factor B	7 (100)	—	—
Lymphocytes + medium	—	10	—
Lymphocytes + factor b	—	10	—
Monocytes + lymphocytes + medium	—	11	12
Monocytes + lymphocytes + factor B	—	22 (77)	24 (73)

Three separate experiments demonstrating inhibition of factor B-induced monocyte-mediated cytotoxicity by lymphocytes. Peripheral blood monocytes (3×10^5) were added to the designated wells in the presence or absence of 25 μl of factor B (titer 40,000/ml). Mononuclear cells or lymphocytes (1.5×10^5) were added to some of the wells. Percent inhibition of factor B-induced monocyte-mediated cytotoxicity by lymphocytes (given in parentheses) was calculated after subtraction of background ^{51}Cr release. Control experiments with a human B cell line (RPMI 1056) were also performed in separate experiments to control for potential crowding and gave 4%, 2%, and 0% suppression.

indicate that, in this type of cytotoxicity, monocytes require factor B, whereas lymphocytes require factor B and, in addition, at least C5, C7, and C8.

Purified Factor B Induces Cytotoxicity by Monocytes but Not by Lymphocytes. These results suggested that purified factor B by itself might support cytotoxicity by monocyte-enriched cell populations. As seen in Fig. 2 a, purified monocytes were, in fact, induced to become cytotoxic in the presence of purified factor B alone (the assay system being

otherwise free of other added serum factors). In contrast, purified factor B alone failed to support cytolysis mediated by lymphocytes (Fig. 2a and Table IV). Fig. 2b shows a dose-response curve for the induction of monocyte-mediated cytotoxicity by factor B. As little as 3 μl of the factor B solution (titer 40,000) was capable of inducing measurable monocyte-mediated target cell lysis under these conditions (3×10^5 monocytes; target/effector ratio of 1:10).

Lymphocytes Inhibit Factor B-induced Monocyte-mediated Cytotoxicity. The demonstration that factor B alone induced monocytes to become cytotoxic was unexpected because we had repeatedly observed that factor B failed to induce whole mononuclear cell preparations to become cytotoxic and because whole mononuclear cells contain 15–40% monocytes. We therefore tested for the possibility that lymphocytes were inhibiting factor B-induced monocyte-mediated cytotoxicity. Table IV shows that freshly obtained lymphocytes inhibited factor B-dependent monocyte-mediated killing 73–100% at a monocyte/lymphocyte ratio of 2:1. As a control for nonspecific effects of higher cell concentrations in these cultures, a human B cell line (RPMI 1056) was also added in a series of control experiments, and in no case was any inhibition observed. We are currently further characterizing the nature of this inhibitory effect.

Discussion

Previously, we showed (1, 2) that human mononuclear cells can lyse xenogeneic erythrocyte target cells in the presence of heat-labile serum factors. In the present report, we further characterized this type of cytotoxicity and presented data that serum complement components cooperate with mononuclear leukocytes in the lysis of target cells. We report a series of experiments with both whole mononuclear cell effector populations and populations enriched in monocytes or lymphocytes. The serum requirements for cooperative cytotoxicity have been dissected using sera deficient in (or depleted of) single complement components as well as purified complement components. By this strategy, we found that, although lymphocytes and monocytes are both capable of cooperating with serum factors in mediating target lysis, the serum factor requirements are different for each type of effector cell.

Monocytes appear to require only added factor B to lyse chicken erythrocyte targets. This conclusion is based on several pieces of data. First, we had previously shown that antibodies to factor B markedly inhibit this form of cytotoxicity. Second, of all the deficient and depleted sera tested, only factor B-depleted serum failed to cooperate with monocytes in target lysis. Purified factor B can reconstitute the activity in factor B-depleted serum. Finally, purified factor B in conjunction with monocytes leads to target lysis in the absence of other added serum factors. These findings are consistent with previous findings reported by Götze et al. (12, 13) that factor B induces macrophage spreading, a morphologic correlate of macrophage activation.

Lymphocytes appear to require a different set of serum factors to become cytotoxic in this system. Experiments with deficient and depleted sera suggest that factor B, C5, C7, and C8 are all required. C3, C4, and factor D appear not to be required for lymphocyte-mediated target lysis. Factor B alone cannot support lymphocyte-mediated target cell lysis.

Serum deficient in C3 cooperated normally with whole mononuclear cell populations as well as populations enriched in monocytes or lymphocytes, in target lysis. This finding was surprising in light of the central importance of C3 in the complement

cascade. Experiments with monoclonal anti-C3, designed to test for the possibility that trace amounts of C3 were sufficient to support killing, were also consistent with the hypothesis that C3 was not required in this system. Furthermore, as discussed above, purified factor B alone was sufficient to support killing by monocytes.

The mechanism by which different complement components cooperate with both monocytes and lymphocytes in target cell lysis is not known. It is likely, however, that different mechanisms exist for lymphocytes and monocytes. Neither cell type appears to require C3, suggesting a novel mechanism for generation of a new form of complement activity. Because monocytes require only factor B in the generation of cell-mediated cytotoxicity, two of many possible mechanisms include (*a*) binding of factor B to monocyte surface, followed by direct activation of the monocyte to become cytotoxic and (*b*) proteolytic cleavage by factor B or a fragment of factor B of another protein, perhaps associated with effector cell (monocyte) or target cell surface. The mechanism of interaction of lymphocytes with serum complement leading to target lysis similarly is open to speculation. In addition to factor B, certain terminal components are also required, including C5, C7, C8, and possibly others. Of note in this regard are studies by Sundsmo et al. (18, 19), demonstrating the presence of "neoantigenic" determinants specific for the C5b-9 membrane attack complex on peripheral blood leukocytes. This has been postulated to be involved in the expression of cytotoxicity by mononuclear leukocytes. It may be that the system of lymphocyte-mediated cytotoxicity described in the present report involves a similar mechanism of assembly of complement components on the surface of the effector cell. One possible inconsistency, however, is that "neoantigen" was found primarily on non-T cells (18), whereas in our system, lymphocyte-mediated cytotoxicity was found primarily in the T cell (E-rosette positive) population (2).

We have also shown in the present report that, in addition to acting as effector cells in mediating target cell lysis, lymphocytes also may modulate the expression of monocyte-mediated cytotoxicity in vitro. Thus, lymphocytes markedly inhibit (73–100%) target lysis in the presence of monocytes and factor B. The identification of the inhibitory cell as well as the mechanism of inhibition are presently under investigation. Two likely possibilities for the mechanism of inhibition include (*a*) inactivation of factor B by lymphocytes and (*b*) direct suppression of cytotoxic monocytes. The observation that monocytes in the presence of lymphocytes (i.e., unseparated mononuclear cells) fail to kill the target erythrocytes in factor B-containing C5-deficient serum but do kill the targets in sera deficient in C3, C4, C7, and C8 suggests that C5 may play a role in modulating the effects of lymphocytes on monocyte-mediated cytotoxicity.

In conclusion, the present report further characterizes a novel form of cytotoxicity that links two host-defense systems, the serum complement system and mononuclear cells. Among the several unique features of this system is that C3 appears not to be required. Both lymphocytes and monocytes can act as effector cells in this form of cytotoxicity, however, probably through different mechanisms. In addition, lymphocytes can also act in vitro to modulate monocyte-mediated cytotoxicity. Such a system of cytotoxicity would offer an attractive capability for first line host defense against foreign cells in vivo.

Summary

Synergistic cytotoxicity is a term used to describe a cytotoxic system in which xenogeneic erythrocyte target cells are lysed in the presence of nonimmune human

mononuclear effector cells and antibody-depleted normal human serum. Neither the mononuclear cells nor the serum alone are cytolytic to the target erythrocytes. Previous studies have shown that the serum activity is not immunoglobulin and is heat-labile, suggesting a similarity to serum complement. In this report, sera deficient in various complement components as well as highly purified single complement components were tested with whole mononuclear cell populations and purified monocytes and lymphocytes to further characterize this cytotoxicity system. Whole mononuclear cell populations failed to mediate target cell lysis in sera deficient in C5 or factor B. However, C3-deficient serum, even in the presence of anti-C3 antibody, supported synergistic cytotoxicity normally. Purified lymphocytes were also normally cytotoxic in C3-deficient serum but failed to lyse targets in sera deficient in C5, C7, C8, or depleted of factor B. Purified monocytes failed to lyse the target cells only in factor B-depleted serum and could lyse the target cells in serum-free medium when purified factor B alone was added. Monocyte-mediated cytotoxicity induced by factor B was inhibited 73–100% by adding lymphocytes back to the purified monocytes. Thus, both lymphocytes and monocytes can serve as effector cells in this form of cytotoxicity but require cooperative interaction with different sets of complement components. In addition, lymphocytes can modulate the monocyte-mediated form of target cell lysis associated with factor B.

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