ANTIBODY-DEPENDENT CELL-MEDIATED ANTIBACTERIAL ACTIVITY OF HUMAN MONONUCLEAR CELLS

I. K Lymphocytes and Monocytes are Effective against Meningococci in Cooperation with Human Immune Sera

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It has been well demonstrated that lymphocytes can, in the absence of complement, kill a variety of mammalian and fowl target cells in cooperation with specific antitarget cell antibodies in vitro (1, 2). Recent experiments have suggested that this immune process, generally referred to as antibody-dependent cell-mediated cytotoxicity (ADCC),[1] may be an important host immune defense mechanism against infectious agents (3). Specifically, virus-infected cells (4), parasites (5, 6), and bacterial membrane-coated erythrocytes (7) have been used as targets in ADCC reactions. Consequently, we considered it possible that lymphocytes might also have antibody-dependent antibacterial capabilities. Whereas the ability of monocytes (8) and polymorphonuclear leukocytes (PMN) (8, 9) to phagocytize and kill bacteria in cooperation with opsonizing antibody and complement (10) is well known, lymphocyte-mediated antibacterial activity has not previously been well explored.

We have investigated the in vitro cell-mediated antibacterial capability of human peripheral blood lymphocytes and monocytes in the absence of complement. Viable group C meningococci (Mgc) were used as targets in a modified form of an assay previously used to measure the bactericidal activity of monocytes (8, 11). Because the major human effector lymphocyte in ADCC has been identified as a K cell (which possesses readily identifiable Fc receptors but lacks easily detectable surface immunoglobulin) (2, 12–14), we also tested isolated K-, T-, and B-lymphocyte subpopulations in our assay. Our data indicate that in the presence of human immune serum known to contain high levels of antimeningococcal antibody (Ab), lymphocytes (as well as monocytes) can be effector cells mediating antibody-dependent cell-mediated (ADC) antibacterial activity. Furthermore, K cells, but not T or B cells, possess this capability. In the absence of Ab, we could not demonstrate antibacterial activity by any cell type tested.

Materials and Methods

Bacteria. Pathogenic group C serotype II Mgc (*Neisseria meningitidis* strain 138-I) originally isolated from spinal fluid were stored and cultured for use as previously described (15). Just before the antibacterial assay, the organisms were diluted in Mueller-Hinton broth to appropriate concentrations and maintained at 4°C.

Antisera. Postvaccination antisera from normal adults who had antibody rises after immunization with group C meningococcal polysaccharide (Csss) (16) were used at dilutions ranging from 1:5 to 1:1,280. 0.1 ml of immune serum typically contained enough anti-Csss antibody to bind 5,200 ng of Csss measured in the radioactive-binding assay developed by Brandt et al. (17). Also, the Ab used were effective in a standard, complement dependent (cell-free) bactericidal assay (15) at titres up to 1/640–1/1,280. All antisera were heat inactivated at 56°C for ½ h before use to destroy all complement and noncomplement heat-labile opsonins. To adsorb anti-Mgc antibodies, 4 ml of diluted antisera were rotated end-over-end for 2 h at 4°C in 13 × 75-mm test tubes containing an overnight Petri plate growth of PBS-washed homologous Mgc (strain 138-I) or heterologous (group B, serotype 6, strain M990) meningococci. Bacteria were removed by centrifugation and the adsorbed sera sterilized by passage through a 0.45-μm millipore membrane. Adequacy of adsorption of anti-Mgc antibodies from the antiserum was tested using a standard bactericidal assay (15).

Preparation of Enriched Populations of Lymphocytes and Monocytes. Peripheral blood was obtained from either normal adults or from adults who had been successfully immunized with Csss. Mononuclear cells were separated from either heparinized or defibrinated blood by dextran sedimentation followed by Ficoll-Hypaque (Pharmacia Fine Chemicals, Div. of Pharmacia Inc., Piscataway, N. J.) density centrifugation as previously described (18). These mononuclear cells were incubated overnight at 37°C in 5% CO_2 humidified atmosphere in 75 cm² plastic Falcon flasks (Falcon Labware, Div. of Becton, Dickinson & Co., Oxnard, Calif.) at a concentration of 2 × 10⁶ cells/ml. Incubation was performed in culture media (RPMI-1640 with 2.5% Hepes buffer and 1-glutamine brought to pH 7.4 plus 20% heat-inactivated fetal calf serum). During this incubation, contaminating PMN underwent autolysis (2, 19) and the majority of monocytes adhered to the plastic flasks (2–4). Mononuclear cells that did not adhere to plastic were washed three times in culture media and then used in the antibacterial assay. Mononuclear cells that did adhere to plastic after the overnight incubation were bathed in serum-free Hanks' Balanced Salt Solution without Ca^{++} and Mg^{++} for 2 h at 4°C, dislodged from the flasks by vigorous shaking (and, when necessary, use of rubber policemen), washed, and then used without further purification.

Preparation of Purified Populations of Lymphocytes. Nonadherent mononuclear cells were further depleted of monocytes by passage over a nylon wool column as previously described (4, 12, 14).

Preparation of Purified Subpopulations of Lymphocytes. Mononuclear cells were fractionated into lymphocyte subpopulations consisting of T, B, and Null (K) cells using the methods described by Chess et al. (20) and modified as described in part by Nelson and MacDermott (21) (see diagram in Fig. 1).

Identification of Leukocyte (WBC) Populations. Lymphocytes, monocytes, and PMN in each cell population were identified by four previously described methods: (*a*) Wright's stain, (*b*) methylene blue wet mount morphology, (*c*) 0.81-μm latex particle phagocytosis, and (*d*) Kaplow's myeloperoxidase (22) or α-naphthyl butyrate nonspecific esterase (23) stains. The initial Ficoll-Hypaque-separated mononuclear cells consisted of 77.5 ± 7% lymphocytes, 21 ± 8% monocytes, and 1.5 ± 0.5% PMN. The percentage of lymphocytes in each of the populations used in this study was as follows: nonadherent mononuclear cells, 95 ± 3%; purified lymphocytes, 99.5 ± 0.5%; adherent mononuclear cells, 13 ± 8%. The remainder of the cells in each of these populations were morphologically and histochemically monocytes although a somewhat lower percentage of these cells were actively phagocytic. There were no PMN in any mononuclear cell population used. No latex phagocytizing cells were seen in any purified lymphocyte preparation.

The purified T-lymphocyte population did not bear surface immunoglobulin (<0.5%) and 76% formed overnight E rosettes. Isolated B lymphocytes did not form E rosettes (<0.5%) but did bear surface immunoglobulin (86–96%). Null cells did not bear surface immunoglobulin (<0.5%) and did not form overnight E rosettes (<0.5%).
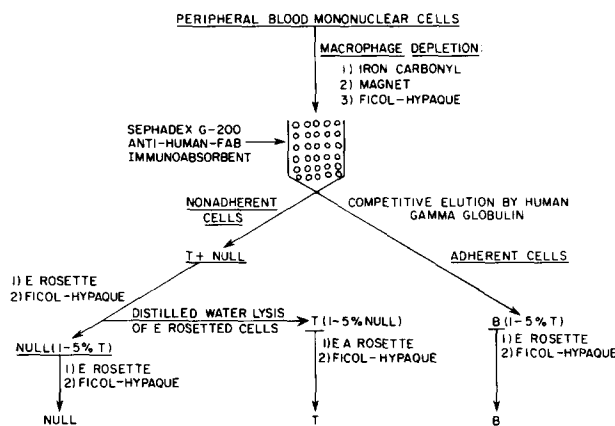
FIG. 1. Isolation of T, B, and Null (K) lymphocytes. We note that the investigators who orginally described this technique have referred to non-surface-immunoglobulin-bearing, non-E-rosette forming, Fc-receptor-bearing lymphocytes as Null cells. We have therefore used the terms "Null cell" and "K cell" interchangeably in this paper.

Cell-Mediated Antibacterial Assay. A modification of the bactericidal assay reported by Steig-bigel et al. (8) and Kretschmer et al. (11) which measures leukocyte-mediated interference with bacterial growth was used to evaluate the antibacterial activity of nonadherent mononuclear cells, purified lymphocytes, and enriched populations of monocytes. The test was performed in 12 × 75-mm sterile glass or plastic tubes into which 0.1 ml of the bacterial suspension (with 2–6 × 10^3 Mgc) were deposited along with 0.1 ml of culture-media-diluted Ab. The tubes, maintained at 4°C, were then centrifuged at 2,400 g for 10 min; 0.2 ml of one of the above WBC populations (containing 0.2-300 × 10^4 WBC) was then added to these tubes. The final volume of each tube was brought to 0.5 ml with cold culture media and the tubes were recentrifuged at 250 g at 4°C for 10 min to maximize contact between WBC and bacteria as recommended by Territo and Cline (24). Control tubes containing the same concentrations of culture media, Ab, and bacteria, but without leukocytes, were treated in a manner identical to and in parallel with the experimental tubes. In experiments designed to conserve reagents and cells, the entire assay was performed in a total vol of 0.1 ml. When this was the case, all quantities in the test tubes were reduced by one-fifth of that described above so that proper concentrations could be maintained. To determine the number of bacteria added to the tubes at the start of each experiment, 20-μl aliquots were taken from sonicated and resuspended representative tubes and plated as described below. The test incubation was then performed at 37°C in 5% CO_2 humidified atmosphere for times varying from 5 to 180 (usually 45-60) min. The tubes were then returned to 4°C and sonicated for 15 s in a cup horn adaptor (model 431A) at a setting of 5 with a model 350 sonicator (Heat Systems-Ultrasonics, Inc., Plainview, N. Y.). Sonication, as recommended by Steigbigel et al. (8), causes release and dispersion of WBC-associated bacteria and prevents clumping which might otherwise result in falsely low colony counts. Microscope examination of sonicated suspensions showed that at this setting, leukocytes were selectively lysed to the extent that even membrane fragments were not discernible. The Mgc, in contrast, were not killed as determined by comparative plating of samples before and after sonication. After sonication, tubes were vigorously vortexed, and replicate or triplicate 20-μl aliquots from each tube were plated on Mueller-Hinton agar and incubated at 37°C in 5% CO_2. The following day, the number of colonies formed from each aliquot was determined.

Calculation of Antibacterial Activity. To compare the effects of nonadherent mononuclear cells, lymphocytes, and monocytes on Mgc, the data are expressed by the following antibacterial index (ABI): ABI = 100-(100) (No. of viable bacteria in suspensions of bacteria plus WBC)/ No. of viable bacteria in suspensions of bacteria without cells at the same time period. This index, comparing the viability of Mgc cultured in the presence of effector cells with the viability

of Mgc cultured for the same time period in the same media only without effector cells, was previously used by Steigbigel et al. (8) and Kretschmer et al. (11) as a measure of the bactericidal activity of monocytes. We employed this index to determine the antibacterial activity of monocytes and lymphocytes because it corrects for day-to-day variation in bacterial growth as well as any facilitatory or inhibitory influence of the incubation media (including Ab) on meningococcal viability.

Results

ADC Antibacterial Activity of Nonadherent Mononuclear Cells and Purified Lymphocytes. The kinetics of ADC antibacterial activity mediated by either nonadherent mononuclear cells (which contained 5% monocytes) or purified lymphocytes (contaminated with 0.5% monocytes) indicate that effector cell activity was minimal before 15 min and tended to peak after 45 min of test incubation at 37°C (Fig. 2a and b).

Effect of Leukocyte/Bacteria Ratio on ADC Antibacterial Activity. As the leukocyte-to-bacteria ratio increased from 150/1 to 800/1, the ABI induced by either nonadherent mononuclear cells or purified lymphocytes increased concomitantly (Fig. 3a). These data demonstrate that passage of nonadherent mononuclear cells over nylon wool reduces effector capacity 25–30% relative to the antibacterial activities of the cells before nylon wool passage.

ADC Antibacterial Activity of Enriched Populations of Monocytes. As shown in Fig. 3b, at ratios of 30/1, 120/1, and 400/1, monocyte-mediated antibacterial activity averaged 35, 68, and 94%, respectively. At ratios <16/1, however, no significant antibacterial activity could be detected. Because purified lymphocyte populations were contaminated with 0.5% monocytes, even at purified lymphocyte/bacteria ratios which elicited maximum antibacterial activity (800/1), the monocyte/bacteria ratio was only 4/1. Consequently, it is highly unlikely that monocytes contributed to the antibacterial activity mediated by our population of purified lymphocytes. Nonadherent mononuclear cells, in contrast, with 5% monocyte contamination, did contain enough monocytes to account for some of the antibacterial activity mediated by this cell population. For example, when using a nonadherent mononuclear cell/bacteria ratio of 300/1, an average ABI of 64% was found (Fig. 3a); the monocytes in this population could have caused an ABI of 17% (i.e. 26% of the total) because the monocyte/bacteria ratio was 16/1 (Fig. 3b). By this method of calculation, monocytes could have contributed from 26 to 43% of the antibacterial activity mediated by nonadherent mononuclear cells, with lymphocytes effecting the remainder.

ADC Antibacterial Activity of Purified Lymphocyte Subpopulations. To determine which lymphocyte subpopulations were effective in our ADC antibacterial system, purified B, T, and K cells were compared with simultaneously run unseparated lymphocytes (containing T, B, and K cells) and monocytes. The results of seven such experiments, shown in Table I, demonstrate that K cells are the only lymphocytes which are effector cells in our ADC antibacterial system. The antibacterial activity of purified T and B cells was negligible. Moreover, the antibody-dependent antibacterial capacity of K cells was found to be comparable to that of monocytes.

Effect of Prior In Vivo Immunization on Cell-Mediated Antibacterial Activity. Neither nonadherent mononuclear cells nor purified lymphocytes could exhibit any antibacterial activity independently (without Ab) even when cells from immunized individuals were used (data not shown). In addition, enhancement was not detected when
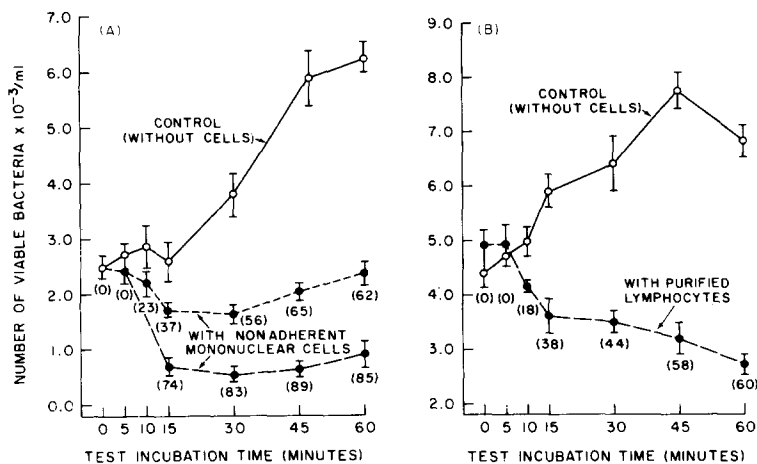
Fig. 2. Kinetics of ADC antibacterial activity. (A), Nonadherent mononuclear cells; (B), purified lymphocytes. Each point represents the mean ± SEM of three values. Numbers in parentheses represent antibacterial indices calculated by the formula shown in Materials and Methods. Antiserum, used at a dilution of 1/160, was constant ●- - -●, leukocyte/bacteria ratio was 400/1; ●———●, leukocyte/bacteria ratio was 800/1.

the ADC antibacterial capacity of nonadherent mononuclear cells from immunized individuals (72 ± 4%) was compared to that of nonimmunized persons' cells (76 ± 10%). Similarly, ABI elicited by purified lymphocytes of immunized persons (63 ± 18%) and nonimmunized individuals (53 ± 14%) were not different from one another.

Effect of Altered Physiologic Conditions on ADC Antibacterial Activity. The inability of mononuclear cells to mediate ADC antibacterial activity under altered physiologic conditions was determined by two methods: (a) No cell population could elicit any significant antibacterial activity when the entire test was performed at 4°C (Table II). (b) When mononuclear cell populations were pretreated at 46°C for 15–30 min before their use in our standard assay (run at 37°C), their ability to mediate ADC antibacterial activity was essentially abolished (Table II). Cell viability, as measured by trypan blue exclusion, was not altered by either heat pretreatment or maintenance at 4°C.

Effect of Ab Concentration on ADC Antibacterial Activity. The concentration of immune sera which elicited optimal nonadherent mononuclear cell-mediated (Fig. 4) or purified lymphocyte-mediated (not shown) antibacterial activity was generally 1/160. For most immune sera, significant ABI could still be detected using serum dilutions up to 1/640 or 1/1,280. In contrast, when normal adult sera were used, the optimal serum dilution was usually 1/10 and significant antibacterial activity was generally not present at dilutions >1/80 (not shown). When no serum was present, the antibacterial index was invariably insignificant. Fig. 4 also depicts a prozone phenomenon of decreased antibacterial activity frequently found when supra-optimal concentrations of Ab were used.

Effect of Adsorption of Specific Antibody from Antisera on the Ability of Sera to Induce Cell-Mediated Antibacterial Activity. Adsorption of antisera with homologous Mgc reduced purified lymphocyte-mediated antibacterial activity from 51 ± 7% to 0 ± 3%. Complement-mediated (cell-free) bactericidal activity was reduced from 98 ± 1% to
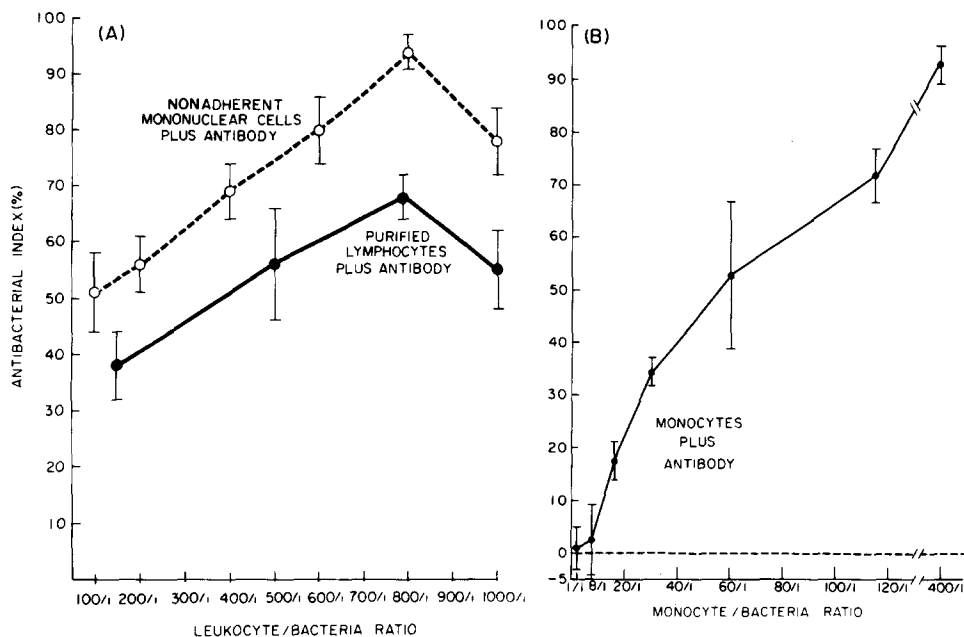
Fig. 3.   Effect of leukocyte/bacteria ratio on ADC antibacterial activity. (A), Nonadherent mononuclear cells and purified lymphocytes; (B), enriched populations of monocytes. Each point represents the mean ± SEM of three to eight experiments. Antiserum, used at a dilution of 1/160, was constant. Antibacterial indices were calculated by the formula shown in Materials and Methods.

9 ± 10%, thus indicating that all functional anti-138-I meningococcal antibodies had been removed. When heterologous meningococci (strains M990) were used in the adsorption, both cell-mediated and (cell-free) ADC antibacterial activity were retained (ABI equalled 67 ± 9% and 98 ± 2%, respectively). Identical results were obtained using nonadherent mononuclear cells.

Discussion

The data presented in this study demonstrate that normal human peripheral blood lymphocytes (specifically, K cells) have the capacity (as do monocytes) for ADC antibacterial activity against Mgc in cooperation with human heat-inactivated anti-Csss immune serum. Lymphocyte-mediated immunity to infectious agents has previously been demonstrated using parasites (5, 6), virus-infected cells (4, 13), or bacterial membrane-coated erythrocytes (7) as targets in ADCC assays. Our study, using replicating meningococci as targets extends the antibody-dependent effector capacity of K lymphocytes to bacteria.

In previously described ADCC systems, target cell lysis has been measured by release of radiolabeled material from damaged target cells. Because in our system we measure the number of viable target bacteria by their ability to form colonies after overnight culture on agar, the mechanism of lymphocyte-mediated antibacterial activity may be different from that of classically described ADCC. Nevertheless, our ADC antibacterial system is similar to ADCC in a number of aspects as follows: (a) The major lymphocytic effector cell in both systems is a K cell (12-14). (b) Altering

TABLE I

Comparison of ADC Antibacterial Activity of Purified Lymphocyte Subpopulations and Monocytes

Cell type	Antibacterial index*	
	WBC/bacteria ratio.	
	400/1	100/1
Lymphocytes (T, B, and K)	43 ± 14	31 ± 6
B cells	11 ± 4	18 ± 6
T cells	6 ± 6	12 ± 6
K cells	67 ± 18	59 ± 8
Monocytes	83 ± 10	53 ± 17

* Antibacterial index was calculated as described in Materials and Methods.

physiologic conditions by either preheating the effector cells at 46°C (25) or performing the entire assay at 4°C (25, 26) abrogates both ADCC and ADC antibacterial activity. (c) Neither in vivo nor in vitro immunization is necessary for effector cell activity of either type. Moreover, cells from immunized persons do not exhibit enhanced activity compared to cells from nonimmunized individuals. Using *Shigella flexneri* 2a as targets, MacDermott et al.[2] have similarly found that the antibody-dependent antibacterial activity of cells from individuals actively infected with shigellae was not different from that of uninfected persons' cells. (d) As in all ADCC systems, immune specificity is totally dependent on the effect of specific antitarget cell antibodies; indeed, the "prozone" phenomenon of decreased activity in the presence of supra-optimal amounts of antibody has also been described in ADCC (1). (e) Scornik has shown that although measurement of release of radiolabeled material from damaged cells may not be detectable for many hours, the critical lytic event in ADCC can occur from 10 to 15 min after initiation of effector and target cell contact (26). Similarly, although we measure the ability of viable bacteria to form colonies after 18 h, ADC antibacterial activity can be demonstrated only when effector cells and target bacteria are together for at least 15 min.

These similarities, however, do not prove that the ADC antibacterial mechanism that we have described is identical to that of ADCC. One of the hallmarks of ADCC is that effector cells exert their cytotoxicity extracellularly. While it is relatively straightforward to determine that target erythrocytes or nucleated cells are extracellular to effector cells, this distinction is more complex when the target cells are bacteria. Specifically, without electron microscope studies, it is difficult to determine whether bacteria are in or on effector cells. Although our purified lymphocyte populations did not phagocytize 0.81-μm latex particles, because certain lymphocytes may acquire increased capacities for endocytosis after in vitro incubation (27), it may be premature, at present, to assume that even our purified lymphocyte populations effect their antibacterial action via an extracellular mechanism. Similarly, because monocytes are capable of both ADCC (28) and phagocytosis (8), we do speculate at this time as to the mechanism whereby monocytes are effective in our system.

[2] MacDermott, R. P., Jr., G. H. Lowell, P. L. Summers, M. Bertovich, G. Nash, A. A. Reeder, and S. B. Formal. 1979. Antibody-dependent cell-mediated antibacterial activity of human mononuclear cells. III. Anti-shigella activity of lymphocytes, monocytes and granulocytes. Submitted for publication.

TABLE II

Effect of Altered Physiologic Conditions on ADC Antibacterial Activity

Cell type	Antibacterial index*		
	Regular assay	Assay run at 4°C	WBC pre-incubated at 46°C for 15–30 min before use in regular assay
Nonadherent mononuclear cells‡	61 ± 5	5 ± 4	12 ± 9
Purified lymphocytes‡	50 ± 5	9 ± 8	17 ± 6
Monocytes§	70 ± 10	13 ± 7	18 ± 4

* Antibacterial index was calculated as described in Materials and Methods.
‡ Leukocyte/bacteria ratio was 500/1.
§ Monocyte/bacteria ratio was 200/1.

We estimated the antibacterial contribution of the 5% monocytes in our nonadherent mononuclear cell population to be ~25–36% (see two methods of calculation in Results section). The percentage of K cells among peripheral blood lymphocytes has been shown to average ~14% (14, 29). By adding the 5% monocytes to these figures, it can be seen that 19% of the nonadherent mononuclear cells have effector cell potential. It follows that although monocytes constitute only 5% of the total number of nonadherent mononuclear cells, they represent ~26% (5/19) of the total number of effector cells (K cells plus monocytes) in this population. It is therefore not surprising that the antibacterial activity generated by nonadherent cells reflects activity mediated by both monocytes and K lymphocytes.

Although monocytes have been reported to be bactericidal at monocyte/bacteria ratios substantially lower than ours (8), it is probable that the absence of 10% fresh serum as a complement source in our test necessitated our use of more effector cells. Indeed, as shown by Steigbigel et al. (8) heat inactivation of sera (as performed in our experiments) reduces the bactericidal capacity of human monocytes for gram negative bacteria by >90%.

Immune specificity in our system is dependent solely upon the presence of antibodies which are directed against the target bacteria. Thus, all antibacterial activity was eliminated when immune sera were adsorbed with homologous (but not heterologous) Mgc. Moreover, recent data from our laboratory indicate that IgG purified from human immune serum is indeed effective in our system.[3] The critical role of specific antibodies in lymphocyte and monocyte-mediated antibacterial activity against Mgc is not surprising because susceptibility to meningococcal disease has been correlated in humans with the absence of antibody-mediated bactericidal activity (30). Furthermore, immunization of humans with Csss results in (a) protection against group C meningococcal disease (31) and (b) the development of antibodies which can be bactericidal in cooperation with either complement (16) or human peripheral blood PMN (9). Our demonstration in this study that human Csss-immune sera can
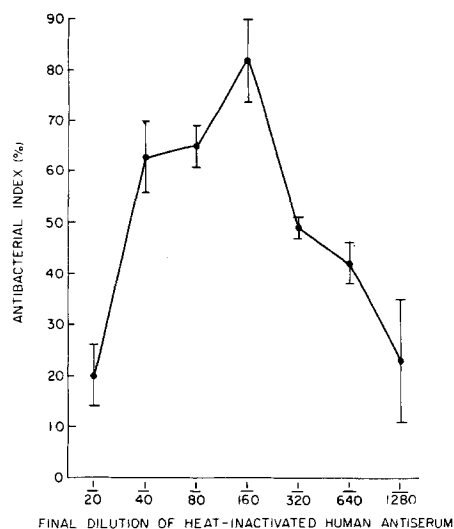
FIG. 4. Effect of antiserum dilution on antibody-dependent nonadherent mononuclear cell-mediated antibacterial activity. Each point represents the mean ± SEM of three experiments. The leukocyte/bacteria ratio, 400/1, was constant. Antibacterial indices were calculated as described in Materials and Methods.

cooperate with lymphocytes and monocytes in the absence of complement extends the mechanism by which acquired anti-Mgc antibodies can be effectively antibacterial.

It is difficult to determine to what degree and in which physiological setting each of the above antibacterial mechanisms contribute to host immune defense. Because lymphocyte- and monocyte-mediated ADC antibacterial activity occurs in the absence of complement, this mechanism may be important in the elimination of bacteria from those areas relatively deficient in physiologically active complement such as cerebrospinal fluid (32) and mucous membrane secretions (33). Indeed, respiratory mononuclear cells have already been shown to mediate secretory IgG- (and to a lesser extent, IgA-) dependent complement-independent antibacterial activity (34). An appreciation of the role of ADC antibacterial activity in acquired and natural immunity to various bacterial diseases requires human epidemiologic studies with the appropriate bacteria and human sera. Our data suggest that such investigations may be fruitful.

Summary

In cooperation with human heat-inactivated antisera from adults immunized with group C meningococcal polysaccharide, normal human peripheral blood mononuclear cells significantly decreased the viability of group C meningococci (Mgc) in vitro. K lymphocytes (Null cells) and monocytes, (but not T or B lymphocytes) were capable of effecting antibody-dependent cell-mediated (ADC) antibacterial activity in this system. The degree to which meningococcal viability was decreased was a function of the length of the test incubation, the concentration of effector cells, and the amount of antiserum used in the assay. When specific antibodies directed against Mgc were adsorbed from the antiserum, cell-mediated antibacterial activity was abolished. ADC

antibacterial activity was also abrogated by performing the assay at 4°C or by heating effector cells to 46°C for 15 min before the assay. Similarities between this ADC antibacterial system and previously described ADCC assays are discussed. The data suggest that K cells (as well as monocytes) may play a role in host immune defense against pathogenic bacteria.

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