

BINDING OF AGGREGATED γ -GLOBULIN TO ACTIVATED T LYMPHOCYTES IN THE GUINEA PIG

BY JOHN A. VAN BOXEL AND DAVID L. ROSENSTREICH

(From the Arthritis and Rheumatism Branch, National Institute of Arthritis, Metabolism, and Digestive Diseases and the Laboratory of Microbiology and Immunology, National Institute of Dental Research, National Institutes of Health, Bethesda, Maryland 20014)

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Aggregated γ -globulin has recently been shown to bind to the surface membrane of thymus-independent, bone marrow-derived "B" lymphocytes and is employed as a marker for this class of cell (1, 2). Membrane receptor sites for the Fc portion of the γ -globulin molecule are believed to be responsible for the binding of such heat-aggregated γ -globulin as well as the binding of antigen-antibody complexes (3).

In this report we demonstrate that a subclass of thymus-dependent "T" lymphocytes in the guinea pig also binds aggregated γ -globulin. Furthermore, we provide evidence that cells of this subclass are activated T cells. Thus not only were such cells found in high proportion amongst peritoneal exudate lymphocytes which constitute a population of activated T cells, but this capacity was shown to develop in immune T cells in vitro when they were activated by antigen.

Materials and Methods

Animals and Immunization.—Male strain 13 guinea pigs weighing 350–400 g (Div. of Research Services, NIH) were immunized in each footpad with 0.1 ml of an emulsion of dinitrophenylated ovalbumin (DNP₁₄-OVA)¹ (250 μ g/ml) in complete Freund's adjuvant. All cells used were from immunized guinea pigs and obtained 2–4 wk after immunization.

Cell Collection and Preparation.—Peritoneal exudate lymphocytes (PELs) were separated from a mineral oil-induced (Drakeol G-VR, Pennsylvania Refining Co., Butler, Pa.) exudate cell population by passing them over a column of nylon-wool and fine glass beads using a previously described technique (4). In order to label phagocytic cells the PEL population was incubated in some experiments with latex beads (1.1 μ m diameter, Dow Chemical U.S.A., Membrane Systems Div., Midland, Mich.) (5). Single cell suspensions of thymus were sub-

¹ *Abbreviations used in this paper:* Agg+ cells, cells binding heat-aggregated guinea pig γ -globulin; DNP-OVA, dinitrophenylated ovalbumin; EAC, sheep erythrocytes sensitized with antibody and complement; E-rosette+ cells, cells forming spontaneous rosettes with rabbit erythrocytes; FCS, fetal calf serum; flu-anti-Fab, fluorescein-conjugated rabbit anti-guinea pig Fab; Ig+ cells, cells staining for surface Ig; PBS, phosphate-buffered saline; PELs, peritoneal exudate lymphocytes; rho-anti-Ig, rhodamine-conjugated rabbit anti-guinea pig Ig.

jected to an identical column purification procedure and single cell suspensions of draining lymph node were treated by such column purification where indicated. Contaminating erythrocytes in these preparations were lysed with isotonic ammonium chloride (6).

Antisera.—Antiguinea pig immunoglobulin (Ig) and antiguinea pig Fab antisera were prepared by immunization of rabbits with the γ_2 -fraction or the Fab fraction of guinea pig γ -globulin, respectively (these antisera were a gift of Dr. V. Nussenzweig, New York University).

Antiserums were precipitated twice with 40% ammonium sulfate and conjugated with fluorescein isothiocyanate or tetramethyl-rhodamine isothiocyanate (Bio Quest Div., Becton, Dickinson & Co., Cockeysville, Md.) (7). Free fluorescein was removed by filtration through G-25 Sephadex (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.). These antisera were centrifuged at 2,500 rpm for 5–10 min immediately before use, to remove insoluble material.

Identification of Cells Binding Aggregated γ -Globulin.—The 7S fraction of guinea pig γ -globulin (Schwarz/Mann Div., Becton, Dickinson & Co., Rockville, Md.) was dissolved in phosphate-buffered saline (PBS) at 50 mg/ml and heated at 63°C for 15 min (1, 8). The aggregates were homogenized in PBS and centrifuged at 1,000 g for 30 min and the supernate used. The aggregate preparation was incubated with cells at various dilutions to determine the concentration that stained the cells optimally. The final protein concentration used in these experiments was 0.04 mg/ml. The preparation was stored at -20°C and portions thawed before use.

5 million viable lymphocytes were incubated for 30 min at 4°C in a final volume of 0.1 ml, with 10 μl of aggregated guinea pig γ -globulin in RPMI 1640 medium (Grand Island Biological Co., Grand Island, N. Y.) with 0.02% sodium azide. Cells were gently resuspended every 10 min. They were then washed through fetal calf serum (FCS) (Microbiological Associates, Inc., Bethesda, Md.) and again with medium containing 5% FCS at 4°C , and the pellet incubated at 4°C with 0.1 ml of undiluted rhodamine-conjugated anti-Ig antiserum (rho-anti-Ig) for 30 min. The cells were then again washed through FCS and after a final wash in medium mounted in suspension on slides with vaseline-sealed coverslips and examined with a Leitz Ortholux microscope with an Osram HBO 200 mercury-arc lamp and a Leitz vertical illuminator (E. Leitz, Inc., Rockleigh, N. J.) (9). This indirect-staining technique was found to be more sensitive than the direct method using fluorochrome-labeled aggregates. Lymphocytes were first identified under tungsten bright-field illumination and the same field was then examined by ultraviolet light so that the fraction of lymphocytes with fluorescent aggregates could be determined. At least 200–300 lymphocytes were counted in each preparation.

Identification of Ig-Bearing Cells.—5 million viable lymphocytes were incubated at 4°C in 0.1 ml of undiluted fluorescein or rhodamine-conjugated antiserum in the presence of 0.1% sodium azide for 30 min. They were washed through FCS and then in medium, and mounted on coverslips. In some experiments the fluorescein-conjugated anti-Fab antiserum (flu-anti-Fab) and rho-anti-Ig were ultracentrifuged at 100,000 g for 60 min just before use to remove any spontaneously aggregated γ -globulin present in the fluorochrome-labeled antisera.

Identification of Cells Forming EAC Rosettes.—Rosettes were formed with sheep erythrocytes (E) sensitized with the 19S fraction of rabbit antish sheep erythrocyte antiserum (A), and mouse complement (C) by the method of Lay and Nussenzweig (10). Lymphocytes with a C receptor identified in this way are B lymphocytes (11).

Identification of T Lymphocytes by Rosette Formation with Rabbit Erythrocytes.—Rosettes were prepared using a modification of previously described techniques (12, 13). Erythrocytes (E) from a freshly bled rabbit were washed four times in PBS and 4×10^7 cells were incubated with 4×10^6 lymphocytes in a total volume of 1 ml of RPMI 1640 (GIBCO) supplemented with 5% FCS for 20 min at 37°C . The FCS used was preabsorbed with rabbit erythrocytes to remove naturally occurring antibody which otherwise tended to cause clumping. The cells were pelleted at 200 g at room temperature and incubated on ice for 1 h. The supernate was

aspirated and the cells gently resuspended in about 50 μ l of medium and mounted on Vaseline edged coverslips. Clusters were counted as rosettes if more than three erythrocytes attached. However, the majority of rosettes bound eight or more erythrocytes (Fig. 1).

Triple-Labeling Experiments.—In some experiments cell populations were first stained with ultracentrifuged fluorescein-conjugated anti-Fab, then washed and incubated with aggregated γ -globulin. After further washing the cells were then stained with rhodamine-conjugated antiserum to the cell-bound aggregates. Finally, the cells were allowed to form rosettes with rabbit erythrocytes. At each stage a portion of the cell suspension was mounted on glass slides and the fraction of cells with one or more markers enumerated in each field with the use of tungsten followed by ultraviolet illumination of the appropriate wavelengths to detect fluorescein or rhodamine staining.

Antigen-Induced In Vitro Lymphocyte Transformation.—Column-purified lymph node lymphocytes were resuspended at a concentration of 2×10^6 cells/ml in tissue culture medium (RPMI 1640 containing: penicillin, 100 U/ml; streptomycin, 100 μ g/ml; glutamine, 2 mM; and 5% heat-inactivated FCS (Industrial Biological Laboratories, Rockville, Md.). They were then reconstituted with 20% irradiated (2,500 R) syngeneic peritoneal exudate macrophages and 1-ml aliquots cultured in glass vials alone or in the presence of DNP-OVA (10 μ g/ml) for 120 or 144 h. At the end of the culture period triplicate vials received a 4-h pulse with 4 μ Ci of [3 H]thymidine (6.7 Ci/mmol; New England Nuclear, Boston, Mass.), and the cells were then harvested and counted as previously described (14). The remainder of the cells were pooled, washed, and tested for surface membrane markers.

RESULTS

Frequency of Agg+ Ig- Lymphocytes in Various Lymphoid Populations.—When lymph node lymphocytes were tested the frequency of cells binding heat-aggregated guinea pig γ -globulin (Agg+) was found to be consistently greater than the frequency of Ig-bearing cells (Ig+). As both aggregate binding and surface Ig are B-cell markers, double-labeling experiments were performed in order to elucidate this discrepancy. Lymph node cells were therefore first labeled with flu-anti-Fab that had been ultracentrifuged to remove spontaneously formed aggregates (green fluorescence). They were then washed and labeled with aggregates using rho-anti-Ig (red fluorescence). It was found that approximately 10% of such cells bore rhodamine-labeled aggregates but failed to stain for surface Ig. This Agg+ Ig- population could therefore represent Ig- B cells or Agg+ T cells.

In order to determine if T lymphocytes could bind aggregates we examined two T-cell populations, thymus cells and PELs, for the occurrence of such Agg+ Ig- cells. PELs are column-purified peritoneal exudate cells that consist of greater than 90% T lymphocytes and only 1-2% B lymphocytes (15). In each experiment lymphocytes from lymph node, peritoneal exudate, and thymus were treated identically in one of two ways: (a) Aggregates followed by rho-anti-Ig (aggregate-binding cells) or (b) rho-anti-Ig alone (Ig-bearing cells). The lymph node population had 32% Agg+ cells but only 22% Ig+ cells (Table I). Using rosette formation with complement-coated sheep erythrocytes (19S EAC) as an alternate B-cell marker, similar results were obtained: 31% of cells were Agg+ and only 24% formed EAC rosettes. A strikingly high proportion of Agg-binding lymphocytes was found amongst PELs. Thus PELs

had 59% Agg+ cells but less than 1% Ig+ cells. In contrast thymus cell populations had only 1.5% Agg+ and <1% Ig-bearing cells. Since PELs are an almost pure population of T cells, these data provide strong evidence that a subpopulation of T cells can bind aggregated γ -globulin.

Populations with Agg+ Ig- Cells Allowed to Form Spontaneous E-Rosettes.—To confirm that these cells are T cells, and to rule out the possibility that the Agg+ Ig- cells found in the lymph node represent Ig- B cells, a T-cell marker was used. Rabbit erythrocytes bind to guinea pig T cells forming spontaneous E-rosettes (12, 13). Experiments were performed to confirm the specificity of this marker (Table II). With a modified technique using incubation in the cold, 96% of guinea pig thymus cells and 90% of PELs formed rosettes and none of the cells forming these rosettes were Ig+. In lymph node cell populations 73% formed rosettes and only 1% of the rosettes was Ig+.

Lymph node and PEL populations were therefore tested using this T-cell marker. The cells were sequentially labeled with ultracentrifuged flu-anti-Fab and with aggregates followed by rho-anti-Ig, and lastly allowed to form E-rosettes (Table III). Using such a triple-labeling technique it was found that in lymph node populations a proportion of E-rosette-forming cells also bound

TABLE I
Frequency of Agg+, Ig+ or, EAC-Rosette+ Lymphocytes in Guinea Pig PEL, Thymus, and Lymph Node Cell Populations

Exp.	Frequency of lymphocytes						
	PELs		Thymus cells		Lymph node cells		
	Agg*	Ig	Agg	Ig	Agg	Ig	EAC-rosette
	%		%		%		
1	57	2	1	1	33	19	
2	60	0	2	0	32	24	
3	70	1	1	2	32	22	
4	50	0	2	0	31	25	24
5	57	4	—	—	31	20	24
Mean	58.8	1.4	1.5	0.8	31.8	21.8	24.0

* Criteria for + was the presence of five or more fluorescent areas/cell.

TABLE II
Rosette Formation by Guinea Pig Lymphocytes with Rabbit Erythrocytes

Cell source	Frequency (% of total)*		
	Rosette+	Ig+ rosette+	Ig+
Thymus	96	0	<1
PELs	90	0	2
Lymph node cells	73	1	23

* The results represent the means of two separate experiments.

TABLE III
Triple Labeling of Lymph Node Cells and PELs to Determine Frequency of Cells Binding Aggregated γ -Globulin and Forming E-Rosettes, but Lacking Surface Ig

Exp.	Cell population	Frequency of lymphocytes (%) with				
		Ig	Agg	E-rosettes	Ig+ E-rosettes	Agg+ E-rosettes
1	Lymph node	29	49	72	<1	23
2	Column-purified lymph node	3	13	92	0	11
3	PELs	1	66	90	0	66

aggregates (23%) but did not stain for surface Ig. Lymph node populations which had been column purified had 11% of such Agg+ E-rosette+ cells, even though Ig+ (B) cells had been depleted by this procedure (8). In the PEL population, 66% of cells were Agg+ Ig- E-rosette+.

Macrophages also bind aggregates although they did not form rosettes with rabbit erythrocytes. However, to rule out the possibility that some of the Agg+ E-rosette+ cells were macrophages, the cells were incubated with latex beads and then allowed to bind aggregates and subsequently form E-rosettes. Beads were found to be phagocytosed by only 5% of cells in the PEL population, which in addition had the morphological features of macrophages and a characteristically high density of aggregate binding. No beads were found in any of the E-rosette-forming cells. These data confirm that the Agg+ Ig- cell both in the lymph node as well as in the PEL population is a T cell.

Capacity of T Lymphocytes to Bind Aggregates Upon Activation by Antigen In Vitro.—There is considerable evidence indicating that PELs form a population greatly enriched in activated T cells. The high proportion of Agg+ cells in PEL populations suggested that these cells might be activated T cells. To test this hypothesis, lymphocytes from animals sensitized to DNP-OVA were activated by antigen in vitro.

Column-purified lymph node cells were used in these experiments. As noted only about 10% of such cells are Agg+ Ig-. When these cells were activated by challenge with DNP-OVA a striking increase was found in the frequency of Agg+ Ig- cells. Again, to confirm that such cells were T cells these populations were allowed to form rosettes with rabbit erythrocytes. The frequency of E-rosette+ cells that bound aggregates in such antigen-stimulated cultures was 45%, 54%, and 41% in three separate experiments as compared to less than 10% in controls (Table IV). E-rosette+ cells staining for surface Ig were not found.

In addition it was observed that the majority of Agg+ E-rosette-forming lymphocytes in the stimulated cultures were much larger than similarly labeled cells in control populations, strongly suggesting that these were blast cells. These findings indicate that T lymphocytes when activated by antigen develop the capacity to bind aggregated γ -globulin.

TABLE IV
Frequency of Aggregate-Binding T Lymphocytes after In Vitro Activation of Immune Lymph Node Cells by Antigen

Exp.	Culture period	Lymphocyte proliferation	Frequency* of total E-rosettes positive for:	
			Agg	Ig
	days	(cpm/2 × 10 ⁶ cells)	%	
1	6	control 1,857	9	
		stimulated 48,713	45	0
2	5	control 1,232	—	
		stimulated 58,066	54	0
3	5	control 1,451	11	
		stimulated 45,520	41	0

* Cells were either incubated with aggregates followed by ultracentrifuged rho-anti-Ig (Agg+) or with rho-anti-Ig alone (Ig+). Both preparations were then allowed to form rosettes. The frequency of E-rosettes varied between 70% and 80% in each experiment. In each preparation the rosettes were identified and scored as either Agg+ or alternatively as Ig+. Dead cells were readily identified by their diffuse staining and were not counted.

Morphological Features of Agg+ T Lymphocytes.—The appearance of such aggregate-binding T cells is shown in Fig. 1. Characteristically, the aggregates were less densely distributed and appeared finer than those on Ig+ (B) cells, or on macrophages. This distinction was noted for Agg+ E-rosette+ cells in PEL populations and antigen-stimulated cultures as well as lymph node cell populations.

DISCUSSION

Binding of aggregated γ -globulin to the cell surface membrane via the Fc receptor is currently believed to be a property of B lymphocytes. In this communication we report that certain T lymphocytes also have the capacity to bind aggregated γ -globulin.

Initially it was observed that a substantial proportion of lymph node cells bound aggregates but did not stain for surface Ig. Such Agg+ Ig- cells also formed rosettes with rabbit erythrocytes, a T-cell marker, which strongly suggested that they were a subpopulation of T cells. Although such cells were not found in thymus cell populations, a very large proportion of Agg+ Ig- cells was found amongst guinea pig PELs. This population is an almost pure population of T lymphocytes. Thus the PEL population has previously been found to lack antibody-forming cells, cells with complement receptors, and cells with easily detectable surface Ig, all characteristic of the B-cell line, while responding to the T-cell mitogen, phytohemagglutinin (15, 16). Our data confirmed that in addition to lack of surface Ig more than 95% of these PELs formed E-rosettes with rabbit erythrocytes. Yet more than half (59%) of these cells bound aggregates to their surface membrane. Furthermore, double-labeling experiments again demonstrated that those cells binding rhodamine-conjugated

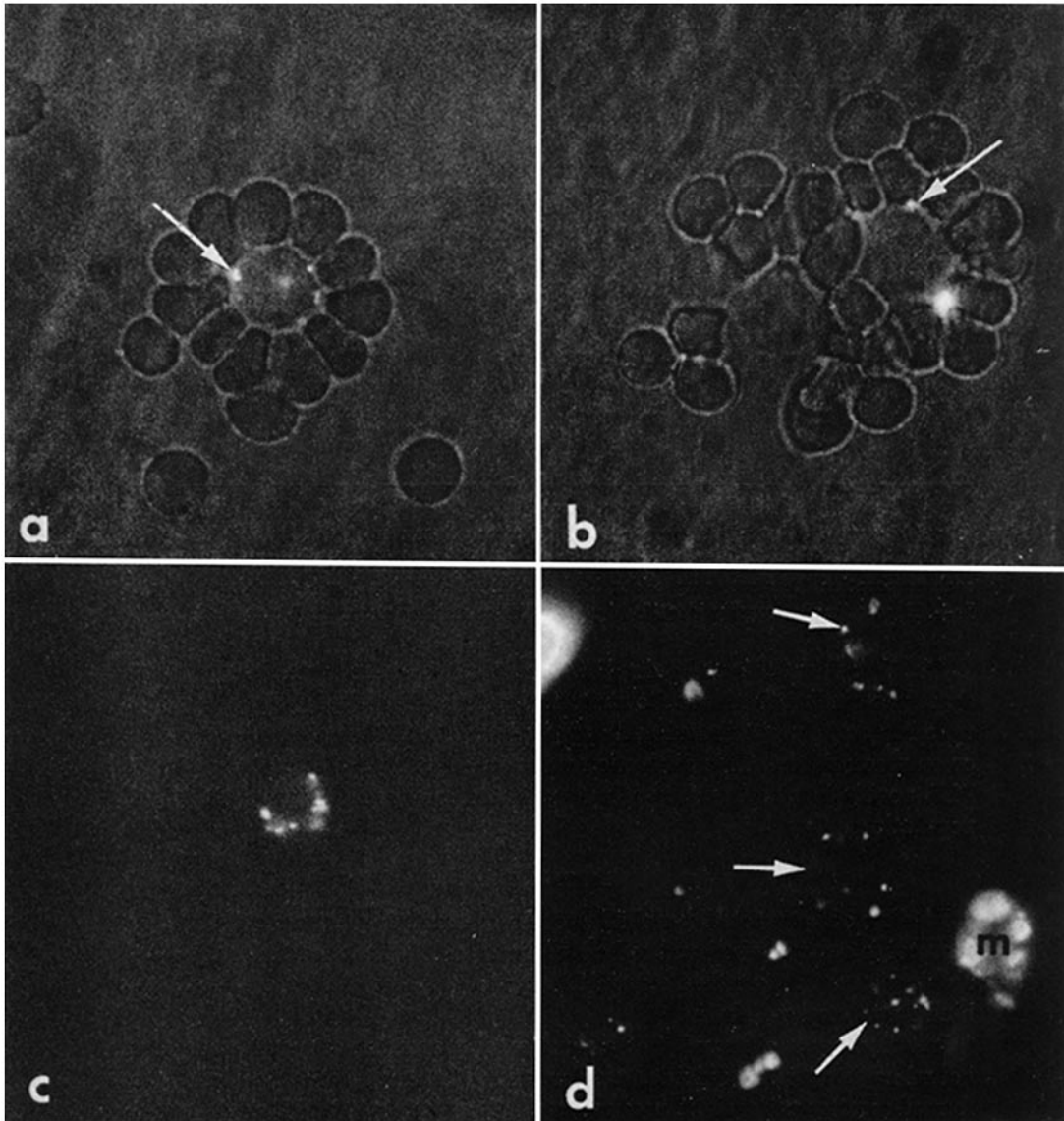


FIG. 1 (a) A PEL double labeled with aggregates and rabbit erythrocytes in the form of an E-rosette (Agg+ E-rosette+ T lymphocyte). About eight aggregates are seen on this cell, arrow points to one of these. (b) Two E-rosette+ lymphocytes. One shows binding of several fluorescent aggregates (arrow points to one of these). The other is a nonaggregate binding T lymphocyte. (c) A lymph node lymphocyte that is densely labeled with aggregates. Cells with this appearance usually stained for surface Ig as well, using a different fluorochrome (B lymphocyte). (d) Three PELs (arrows) with typical fine aggregates less densely distributed than on B lymphocytes or macrophages. On refocusing, other fluorescent aggregates in different focal planes became visible in these cells. Also shown is a macrophage (m) with characteristic dense distribution of aggregates. Cells were photographed either using combined ultraviolet and tungsten illumination (a and b) or ultraviolet illumination alone (c and d).

aggregates also formed E-rosettes but did not stain with ultracentrifuged fluorescein-conjugated anti-Ig, confirming that the aggregate-binding PELs were T cells.

The high frequency of these cells amongst PELs suggested that aggregate binding might be a characteristic of "activated" T lymphocytes, as there is much evidence that the PEL population is greatly enriched in such activated T cells. Thus, McGregor and his coworkers demonstrated that only those lymphocytes that had recently proliferated in response to a new antigenic challenge were able to enter the peritoneal inflammatory site (17-19). Furthermore, PELs have been found to have greatly enhanced reactivity in a variety of experimental situations both *in vivo* and *in vitro*. They possess an enhanced capacity to transfer contact and delayed hypersensitivity (20-22), enhanced capacity to inhibit tumor growth (23), increased ability to produce lymphokines, (5, 24) and a greatly enhanced proliferative response to antigenic challenge *in vitro* (5). Finally, the majority of cells in this population are actively motile, uropod-bearing lymphocytes which is a morphological appearance that correlates with lymphocyte activation *in vivo* and *in vitro* (25-27).

To test the hypothesis that aggregates did in fact bind to activated T cells, we activated sensitized lymph node cells *in vitro* by antigen stimulation and found a striking increase in the percentage of cells that bound aggregates. Double-labeling experiments again showed that these aggregate-binding cells also formed E-rosettes and did not bear surface Ig, and were therefore T cells. A mean of 46% of these Ig- E-rosette+ cells was thus found to bind aggregates, as compared to 10% in control-unstimulated cultures. In addition, the majority of these Agg+ E-rosette+ cells were larger than similarly labeled cells in the control cultures, suggesting that they were blast cells. This finding thus provides confirmatory evidence for the postulate that T cells upon activation have the capacity to bind aggregated γ -globulin. It seems likely, furthermore, that not only aggregate-binding PELs but also aggregate-binding lymph node T cells are activated cells.

The capacity of T cells to bind aggregated γ -globulin upon activation may be due to the development of an Fc receptor on their surface membranes. Nevertheless, although it appears likely that the mechanism of aggregate binding to B and T lymphocytes is the same, this point remains to be established. In this regard, the characteristic pattern of aggregate binding found on these T cells suggests that this aggregate receptor is less densely distributed than on B cells.

Observations made by other workers are consistent with this interpretation. Thus, Yoshida and Anderson reported that mouse thymocytes, activated by passage in irradiated allogeneic mice, developed the ability to bind Ig-coated erythrocytes and were therefore believed to have developed Fc receptors (28). In addition, Grey et al. have reported that a theta-positive mouse lymphoma cell line passively absorbed heat-aggregated IgG2 (29).

The findings in this communication lead to several points of general biological

interest. The ability of activated T cells to bind both aggregated γ -globulin and rabbit erythrocytes may provide a useful marker for the direct identification of these cells in mixed lymphoid populations. Activated T cells could thus be directly identified and quantitated in an inflammatory exudate. The ability of T cells to bind aggregates was demonstrated to develop in vitro when T cells were activated by antigen. A receptor with the capacity to bind aggregated γ -globulin which develops on T-cell membranes upon activation may have fundamental theoretical implications. It may thus augment the binding of activated T cells to target cells in cytotoxic reactions. The possible role of this receptor in antigen T-cell B-cell macrophage interaction in the immune response may be crucial.

SUMMARY

Heat-aggregated guinea pig γ -globulin was shown to bind to the surface membrane of a subclass of guinea pig T lymphocytes. Cells of this subpopulation were identified as T lymphocytes because these cells did not stain for surface Ig (a B-cell marker) but did form spontaneous E-rosettes with rabbit erythrocytes (a T-cell marker). A strikingly high proportion of such aggregate-binding (Agg^+), E-rosette-forming (E-rosette^+), but surface Ig-negative (Ig^-) cells were found in an inflammatory exudate. Thus purified peritoneal exudate lymphocytes (PELs) are known to consist of over 90% T cells, and 59% of these cells bound aggregates. 10% of these Agg^+ Ig^- E-rosette^+ cells were found in draining lymph node cell populations and none in thymus cell populations.

The high frequency amongst PELs suggested that these Aggregate^+ Ig^- E-rosette^+ cells might be activated T cells as these are known to occur in high proportion in PEL populations. Confirmatory evidence for this postulate was provided by the striking increase (from 10% to 46%) of Ig^- E-rosette^+ cells that bound aggregates when lymph node cells were activated by antigen stimulation in vitro.

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