Rosette Formation Assays in Dogs: Lack of Specificity of E Rosettes for T Lymphocytes

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The present study examined the specificity of guinea pig erythrocyte (E) and erythrocyte-antibody-complement (EAC) rosette formation assays with suspensions of canine peripheral blood lymphocytes. Neutrophils, monocytes, and lymphocytes bound EAC but not erythrocyte-antibody (EA) controls. Similarly, all three cell types formed rosettes with guinea pig E. Adherence of guinea pig E to these cells was apparently mediated by natural cytophilic antibodies present in the serum used in the suspension medium. The nonspecificity of the guinea pig E-rosette formation assay with canine lymphocytes renders the technique unreliable for the identification of thymus-derived lymphocytes in dogs.

Identification and quantitation of types of lymphocytes by rosette formation assays is in wide clinical use. Bursa-derived (B) lymphocytes can be identified by using an erythrocyte-antibody-complement (EAC) complex because B lymphocytes possess a surface membrane receptor for activated C3 (2, 15). Thymus-derived (T) lymphocytes can be identified by the presence of differentiation markers such as the theta antigen (13), by lack of surface immunoglobulin, by their ability to form rosettes with macrophages (10), or by the fortuitous presence of a non-immunoglobulin cell membrane receptor for erythrocytes of heterologous or, in some cases, homologous species (6, 14). Of these, the erythrocyte (E) rosette assay is the most popular method because of its simplicity and ease of performance.

The identification of E-rosette-forming cells as T lymphocytes has been established in humans (6), horses (M. J. Tarr, R. G. Olsen, S. Krakowka, G. L. Cockerell, and A. A. Gabel, submitted for publication), pigs (16), cats (17), and dogs (3). In the course of studies designed to evaluate the effects of canine distemper virus infection upon circulating subpopulations of canine lymphocytes, data were obtained suggesting that the guinea pig E-rosette assay is not a reliable T-lymphocyte marker in dogs as previously reported (3). In the present study, the specificity of the E-rosette assay for T lymphocytes was reexamined with lymphocytes obtained from conventional and gnotobiotic dogs.

MATERIALS AND METHODS

Dogs and lymphocyte isolation technique. Colostrum-deprived gnotobiotic dogs were derived by caesarian section from date-mated beagle bitches and raised in flexible plastic isolation units according to the methods of Griesemer and Gibson (4). Specific-pathogen-free conventional dogs were obtained from a colony maintained in the Department of Clinical Sciences, College of Veterinary Medicine, The Ohio State University.

Three to 5 ml of heparinized blood was obtained from each dog, diluted fourfold with Hanks balanced salt solution containing ethylenediaminetetraacetic acid, and submitted to Ficoll-Hypaque gradient isolation by methods described elsewhere (7). The resulting cell suspension containing 70 to 90% mononuclear cells was adjusted to 10^7 cells per ml in complete medium (minimal essential medium containing 20% fetal calf serum [FCS]).

Latex ingestion. A 30% stock suspension of polystyrene beads, 0.794 μm in diameter, in aqueous solution was obtained from Sigma Chemical Co., St. Louis, Mo. A 1:1,200 dilution of stock was made with complete medium, and 3.0 ml of this suspension was added to a cell pellet containing 3 × 10^6 cells. The tubes were agitated at 37°C in a water bath for 45 min.

After this incubation, cells were harvested by centrifugation at 95 × g for 10 min, washed once with Hanks balanced salt solution–ethylenediaminetetraacetic acid, and adjusted to 10^6 cells/ml with complete medium. The number of latex-ingesting cells per 100 cells was determined by survitral staining of a portion with 0.5% new methylene blue in 0.85% saline.

Rosette assays. Sheep erythrocyte-antibody-complement (EAC) rosette formation with Ficoll-Hypaque-purified lymphocytes has been described elsewhere (9). In the present study, cells were reacted with EAC after latex ingestion. For accurate differential counting, a drop of cell suspension stained with new methylene blue was placed on a glass slide, and cells were flattened with a cover slip. This modification of the standard hemocytometer counting technique facilitated identification of rosette-forming cells as lymphocytes, neutrophils,
or latex-ingesting monocytes. EA controls were included in each test.

The E-rosette assay using neuraminidase-treated guinea pig E has also been described (9). Total and differential counts of E-rosette-forming cells were performed after latex ingestion in a manner similar to that for the EAC rosettes.

Effects of serum source and serum absorption with guinea pig E on rosette formation. Lymphocyte rosette formation is dependent upon serum in the suspension medium. For rosette tests, both heterologous and homologous sera in a final concentration of 20% were used. Lots of both FCS and non-E-agglutinating canine serum (CS) were heat inactivated at 56°C for 30 min and then divided into two portions. One portion was saved as an unabsorbed control. The other portion was absorbed with washed packed guinea pig E (0.3 ml of PCV to 1.0 ml of serum) for 12 h at 4°C. The absorbed sera were collected by centrifugation and filtered (0.45 μm) before use. Latex ingestion and rosette formation assays were then performed, using both absorbed and unabsorbed FCS and CS, by the methods described above.

RESULTS

Latex ingestion. One to 12% of mononuclear cells isolated on Ficoll-Hypaque ingested latex beads and were thus identified as monocytes. Occasional neutrophils contained latex beads; however, the lobated nuclear morphology of neutrophils distinguished them from monocytes.

EAC rosettes. The percentages of EAC-rosette-forming peripheral blood leukocytes from 13 gnotobiotic dogs and 8 conventional dogs are given in Table 1. The percentages of EAC-rosette-forming cells ranged from 42 to 79% of the cell suspension tested, with mean value of 59.6 and 55.4% for gnotobiotic and conventional dogs, respectively. This difference was not significant. Differential counts performed on 100 EAC-rosette-forming cells per dog revealed that neutrophils, lymphocytes, and monocytes formed EAC rosettes. Cells tentatively identified as eosinophils were counted as neutrophils, since staining with new methylene blue was not always satisfactory for distinguishing between these cell types.

E rosettes. The percentages of Ficoll-Hypaque-isolated cells forming rosettes with guinea pig E are given in Table 2. A total of 14.8% of cells from gnotobiotic dogs formed E rosettes, whereas 26.9% of cells obtained from conventional dogs did so. As with EAC, neutrophils, lymphocytes, and latex-ingesting monocytes formed E rosettes. Thus only 22.5 to 35.4% of E-rosette-forming cells counted could actually be identified as lymphocytes. The actual range of E-rosette-forming lymphocytes was calculated to be between 3.3 to 9.5% ((E rosettes/100 leukocytes) × (E-rosette-forming lymphocytes/100 E-rosette-forming leukocytes)), levels comparable to that reported by Whitacre and Lang (18).

Effects of serum sources on rosette assays. Before rosette formation experiments, media containing either 10 or 20% FCS or CS were examined for natural agglutinins to guinea pig E under test conditions. By macroscopic examination, FCS was free of agglutinins, whereas CS contained agglutinins. Five of eight CS tested agglutinated guinea pig E. The nonagglutinating sera were from young gnotobiotic dogs.

The presence of natural cytophilic antibodies to guinea pig E in sera would explain the E-rosette-forming ability of neutrophils and monocytes. Accordingly, both FCS and nonagglutinating CS were absorbed with guinea pig E to remove antibodies, and these sera along with unabsorbed control sera were then used as the only serum source in latex ingestion and rosette formation assays (Tables 3 and 4). Absorption of sera with guinea pig E did not affect the percentage of EAC-rosette-forming cells (Table 3). As noted before, differential counts revealed that lymphocytes as well as neutrophils and monocytes formed rosettes with EAC (data not shown).

In contrast to EAC, absorption of sera with

### Table 1. Total and differential counts of EAC-rosette-forming leukocytes isolated by the Ficoll-Hypaque gradient method

<table>
<thead>
<tr>
<th>Dogs</th>
<th>No. of EAC/100 viable leukocytes</th>
<th>Differentiala</th>
<th>Neutrophil</th>
<th>Lymphocyte</th>
<th>Monocyte</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gnotobiotic</td>
<td>(13)</td>
<td>(42-79)d</td>
<td>59.6 ± 10.9c</td>
<td>70.9 ± 16.5</td>
<td>21.5 ± 15.3</td>
</tr>
<tr>
<td>Conventional</td>
<td>(8)</td>
<td>(42-72)</td>
<td>55.4 ± 10.9</td>
<td>57.3 ± 8.0</td>
<td>36.0 ± 7.2</td>
</tr>
</tbody>
</table>

a Differential counts performed on 100 EAC-rosette-forming leukocytes.

b Number of dogs tested.

c Mean ± standard deviation of the mean.

d Range.
TABLE 2. Total and differential counts of E-rosette-forming leukocytes isolated by the Ficoll-Hypaque gradient method

<table>
<thead>
<tr>
<th>Dogs</th>
<th>No. of E/100 viable leukocytes</th>
<th>Differential*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Neutrophils</td>
</tr>
<tr>
<td>Gnotobiotic (13)</td>
<td>14.8 ± 6.1 c</td>
<td>69.5 ± 10.0</td>
</tr>
<tr>
<td>(5-23)</td>
<td>(56-80)</td>
<td>(12-40)</td>
</tr>
<tr>
<td>Conventional (8)</td>
<td>26.9 ± 9.5</td>
<td>54.6 ± 12.4</td>
</tr>
<tr>
<td>(13-40)</td>
<td>(38-72)</td>
<td>(16-56)</td>
</tr>
</tbody>
</table>

* See Table 1.

TABLE 3. Effect of serum absorption with guinea pig erythrocytes upon the percentage of EAC-rosette-forming cells a from three conventional dogs

<table>
<thead>
<tr>
<th>Dog</th>
<th>Fetal calf serum Unabsorbed</th>
<th>Absorbed*</th>
<th>Canine serum Unabsorbed</th>
<th>Absorbed*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>33 c</td>
<td>30</td>
<td>34</td>
<td>35</td>
</tr>
<tr>
<td>2</td>
<td>34</td>
<td>43</td>
<td>37</td>
<td>41</td>
</tr>
<tr>
<td>3</td>
<td>66</td>
<td>63</td>
<td>60</td>
<td>56</td>
</tr>
</tbody>
</table>

* Cells isolated by the Ficoll-Hypaque gradient method.
- Sera absorbed with washed packed guinea pig E at a ratio of 0.3 ml of packed E to 1.0 ml of serum.
- Percentage of rosette-forming cells calculated from number of rosette-forming cells divided by total cells counted × 100.

guinea pig E resulted in a decrease in the percentages of E-rosette-forming cells from approximately 50 to 90% when compared with the number of rosettes in unabsorbed control serum (Table 4). Differential counts of E-rosette-forming cells in absorbed serum revealed that all three cell types were affected by the absorption procedure, since the relative percentages of E-rosette-forming lymphocytes, neutrophils, and monocytes approximated those observed with unabsorbed sera (data not shown).

DISCUSSION

Rosette formation assays with lymphoid cells have great appeal not only for their simplicity but also for the apparent specificity of rosettes for subpopulations of lymphocytes. The present study has examined the specificity of T- and B-lymphocyte rosette formation with Ficoll-Hypaque-purified canine peripheral leukocytes.

A number of investigators have published data on the purity of canine lymphocytes isolated on Hypaque gradients (referenced in 18). The size similarity between neutrophils and lymphocytes may result in significant contamination of the cell preparations with neutrophils. The isolation technique used here results in a cell suspension containing approximately 80% mononuclear cells, with a range of 75 to 95%. Using latex ingestion as a marker for monocytes, 1 to 12% of these mononuclear cells were identified as monocytes. Thus, Ficoll-Hypaque-purified leukocytes contain in reality only 65 to 85% lymphocytes. This degree of purity is acceptable for routine immunological studies such as phytomitogen responses (9), but would not be satisfactory for rosette formation studies if nonlymphoid cells participate in rosette formation unless a method was used that would differentiate between the various leukocytes in suspension.

Following the observations of Bowles et al. (3), in which human and guinea pig E-rosette formation was established as a T-lymphocyte marker in the canine species, a series of experiments was initiated in this laboratory to examine the effects of canine distemper virus infection on canine lymphocyte levels and functions. Initial experiments using methods similar to those of Bowles et al. in which rosettes were enumerated in a hemocytometer confirmed their findings. However, in a related study, canine thymocytes gave low rosette formation values (5 to 20%), and anti-guinea pig E-receptor antisera failed to completely block E-rosette formation with canine lymphocyte preparations (8). That study suggested that the guinea pig E-rosette assay may not be specific for all canine T lymphocytes and prompted a reevaluation of the specificity of canine lymphocyte E-rosette formation.

When rosette-forming cell suspensions were
examined with the cover slip method, EAC-rosette-forming lymphocytes, neutrophils, and monocytes were observed. This was not surprising since these cells possess a surface receptor for the activated third component of complement. When E-rosette-forming cells were examined, not only lymphocytes but also neutrophils and monocytes bound guinea pig E. This observation was unexpected and was in direct conflict with the findings of Bowles et al. (3). Recently, Yang et al., in two separate studies, reported human E-rosette formation with canine venereal sarcoma cells and eosinophils, cells clearly not of lymphoid origin (19, 20).

Taken together, these observations strongly suggest that the E-rosette assay for canine T lymphocytes as reported by Bowles et al. was nonspecific for T lymphocytes and, in fact, that E-rosette-forming ability is not even restricted to cells of lymphoid origin.

If the rosettes formed are not T-lymphocyte specific, a mechanism must exist that would allow binding of E to many different leukocytes and tumor cells. A likely candidate is natural cytoplasmic antibody to guinea pig E in either FCS or CS. These antibodies would bind guinea pig E and attach to lymphocytes, monocytes, neutrophils, eosinophils, basophils, and even platelets, blood elements known to possess an Fc receptor for immunoglobulin. Two lines of evidence were found which supported this hypothesis. CS contains agglutinins to guinea pig E, and they could be demonstrated microscopically under the test conditions. If serum contained agglutinins, it is likely that cells isolated from these animals would have some cytoplasmic antibody absorbed to the surfaces. The second line of support for this hypothesis was developed from results of absorption experiments. Absorption of CS or FCS with guinea pig E removed 50 to 90% of the E-rosette-forming ability of canine peripheral blood leukocytes without affecting the total number or distribution of EAC-rosette-forming cells. The decrease in E-rosette-forming cells was reflected in a proportionate decrease in the number of all three rosette-forming cell types. Thus, the number of E-rosette-forming cells in suspension was dependent upon a serum factor and not upon cell receptors for guinea pig E.

Fc receptors for immunoglobulins are classically restricted to lymphocytes of B-cell lineage (11). Recently, however, a subpopulation of activated murine T cells possessing an Fc receptor has been identified, and biological behavior of these cells has been partially characterized (1, 5). Investigators presented evidence suggesting that Fc-positive and Fc-negative cells represent different subsets of T cells. Thus, in the canine system, it is probable that a portion of lymphocyte-forming E rosettes mediated by cytophilic immunoglobulin are T cells. If this is true, the cellular receptor for E is immunoglobulin and not non-immunoglobulin glycoproteins (12), and Fc-bearing B cells cannot be distinguished from Fc-bearing T cells by the E-rosette formation technique.

In conclusion, the present study has examined the specificity of rosette formation assays in dogs. The data obtained indicate that (i) E-rosette tests for T lymphocytes in the canine species are nonspecific in that neutrophils and monocytes formed rosettes indistinguishable from lymphocytes when counting with a conventional hemocytometer, and (ii) the reaction between canine leukocytes and guinea pig E is mediated by natural cytophilic antibodies. It is possible that E-rosette formation in other species in which specificity is assumed for T lymphocytes may be mediated in part by similar cytophilic antibodies. Further studies should confirm or deny this hypothesis.

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LITERATURE CITED


