THE CLASS OF SURFACE IMMUNOGLOBULIN ON CELLS CARRYING IgG MEMORY IN RAT THORACIC DUCT LYMPH: THE SIZE OF THE SUBPOPULATION MEDIATING IgG MEMORY

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Differing views have been expressed about the class of surface immunoglobulin on the B lymphocytes which initiate secondary responses. Thus, it has been claimed that memory B cells carry the same class of Ig as that secreted by their differentiated progeny (1); that some IgG plasma cells may arise from memory cells bearing surface IgM (2, 3); and that memory cells for IgG and IgA responses possess membrane IgD exclusively (4).

In the previous paper (5) it was shown that the precursors of an IgG response were present in both the lymphocytes bearing a receptor for C3 and membrane immunoglobulin (CR+Ig+)1 and Cr-Ig+ subpopulations of thoracic duct lymphocytes (TDL) from primed rats. It was thought that these subpopulations might reflect two stages in the maturation of memory cells, carrying surface IgM and IgG, respectively. The possibility that the C3 receptor was lost during B-cell maturation was supported by the observation that IgA containing large lymphocytes in rat TDL lacked C3 receptors (5). In the present paper the class of membrane immunoglobulin on memory cells for the IgG response in rats has been examined to establish whether the CR+ and CR− subpopulations carry the same or different classes of surface Ig.

The earlier studies (5) also showed that the CR−Ig+ memory cells, although comprising no more than 1% of all TDL, contributed about 20% of the adoptive IgG response. This result suggested that IgG memory cells were, numerically, a minor population of rat TDL. In the present work the percentage of TDL that mediate IgG memory was determined directly.

Materials and Methods

Animals. Inbred HO (hooded, Ag-B5) rats from a specific pathogen-free colony were used throughout.

Irradiation, Antigens and Immunization, Cells, Plaque-Forming Cell Assays, and Preparation of Rosettes. Irradiation, antigen and immunization, cells, plaque-forming cell assays, and preparation of rosettes were all as described in the previous paper (5). All cell transfers were syngeneic.

1 Abbreviations used in this paper: CR+ and CRL, lymphocytes bearing a receptor for C3; DAB, Dulbecco’s A plus B (phosphate-buffered saline with added calcium and magnesium salts); EAC, sheep erythrocyte-antibody-complement complex; FACS, fluorescence activated cell sorter; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; Ig*, IgA*, IgG*, IgM*, lymphocytes bearing, respectively, immunoglobulin of any class, IgA, IgG, IgM; TDL, thoracic duct lymphocytes.
Rabbit-Antirat Immunoglobulin Reagents. The preparation, purification, and specificity of rabbit F(ab')₂ antibodies to rat Fab, IgA, IgM, and IgG₂ (Fd) have been described elsewhere (6-8). Horse-antirabbit F(ab')₂, prepared as described previously (6) was rendered specific for rabbit F(ab')₂ by absorption and subsequent elution from a rabbit F(ab')₂ Sepharose 4B column. The reagents were the generous gift of Dr. A. F. Williams, Medical Research Council, Immunochimistry Unit, Department of Biochemistry, Oxford University, Oxford, England.

In an independent test of specificity it was found that a fivefold excess of free IgG₂ inhibited by 82% the binding of ¹²⁵I-rabbit F(ab')₂-antirat IgG₂ (Fd) to sheep erythrocytes (SRBC) coated with rat-anti-SRBC antibody. In contrast, purified rat IgM and IgA did not inhibit binding. The binding of ¹²⁵I-horse-antirabbit F(ab')₂ to washed rat lymphocytes, previously incubated with a saturating concentration of rabbit F(ab')₂-antirat Fab, was compared with that to unincubated lymphocytes. The counts bound in the latter case were only 4% of those obtained when preincubated lymphocytes were used.

Iodination of Antibodies. ¹²⁵I (Na ¹²⁵I, Radiochemical Centre, Amersham, England) was coupled to antibodies by a modification (6) of the chloramine-T method of Byrt and Ada (9). The radiolabeled antibodies had specific activities of 7-20 μCi/μg and were diluted in Dulbecco's A plus B (DAB)/10% fetal calf serum (FCS) containing 10 mM sodium azide to 10 μg/ml.

Fluorescence Conjugation. A 2.1% solution in distilled water of anhydrous Na₃HPO₄ was adjusted to pH 9.5 by the addition of NaOH. Fluorescein isothiocyanate (FITC) (Nordic, Maidenhead, England) was added to this buffer to a final concentration of 1 mg/ml and 0.3 ml of this solution added to 1 ml horse-antirabbit F(ab')₂ at 3.8 mg/ml in 0.85% saline. The mixture was incubated at room temperature in the dark for 6 h and separated at 4°C on a G-50 Sephadex column pre-equilibrated with phosphate-buffered saline. The final product had a protein concentration of 1.4 mg/ml and a ratio OD 280/OD 495 nm of 0.95.

Autoradiography. To detect the class of immunoglobulin on lymphocytes bearing complement receptors, TDL were washed twice in DAB/10% FCS and adjusted to a cell concentration of 5 × 10⁶/ml. Four 1-ml aliquots were spun down, the supernatants removed, and individual aliquots resuspended in 100 μl of ¹²⁵I-rabbit F(ab')₂ antibodies against rat Fab, IgA, IgM, or in 100 μl DAB/10% FCS/10 mM sodium azide alone as a control. After 1 h incubation at 4°C the cell suspensions were washed three times in DAB/10% FCS/10 mM sodium azide and resuspended in 0.3 ml of the same medium. Unrosetted complement receptor lymphocytes (CRL) were prepared from the radiolabeled lymphocytes by the addition of an equal volume of sheep erythrocyte-antibody-complement complex (EAC) at 4 × 10⁶/ml (5). The rosette suspensions were diluted with a fivefold excess of FCS and then smeared on to clean glass slides using a large drop of the mixture on each slide. The slides were dried in a current of air, fixed in methanol for 5 min and then dipped in K2 emulsion (Ilford Ltd., Ilford, Essex, England) using a 2:1 mixture of emulsion and distilled water. Autoradiographs were exposed at 4°C for 2 wk. Before developing and fixing, the slides were first immersed for 3 min in an alkaline hardener. To obtain satisfactory staining of both erythrocytes and lymphocytes the developed autoradiographs were stained in two stages (Dr. H. Monié, personal communication). In the first stage the slides were stained for 10 min in a 10% solution of Giemsa in phosphate buffer at pH 6.8. Excess stain was then removed by immersing the slides in 70% ethanol for 3 min and the staining process repeated using 10% Giemsa at pH 5. After a brief rinse in pH 5 phosphate buffer followed by distilled water the slides were dried and cover slips applied.

The class of immunoglobulin on C₃+ and C₃- lymphocytes was also determined by labeling normal and CRL-depleted TDL. Cells were rosetted with SRBC or EAC as described previously (5) and after centrifugation on Isopaque/Ficoll the nonrosetting cells were removed from the upper layer of the gradient, washed, and counted. Aliquots of 5 × 10⁶ cells were then incubated with 100 μl of the various ¹²⁵I-rabbit F(ab')₂ reagents for 1 h at 4°C, washed twice, spun through FCS, and smeared on to subbed slides (10). Autoradiographs were prepared as in the preceding paragraph and stained with Harris' hematoxylin.

A cell was scored as labeled if the grain count was more than twice that of the background count over an equal area of emulsion in the same field. The adoption of this criterion resulted in cells binding more than 2,000-4,000 molecules of ¹²⁵I-rabbit F(ab')₂ reagent being scored positive, a somewhat lower cut off than that adopted by Williams (8, 11).

Isolation on the Fluorescence Activated Cell Sorter (FACS) (12) of Subpopulations of Lymphocytes According to Class of Surface Immunoglobulin. To select cells with surface IgM, 1.4 × 10⁶
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TDL, washed twice in DAB/10% FCS, were pelleted, resuspended in 1.4 ml rabbit F(ab')2-antirat IgM at 14 μg/ml and incubated on ice for 1 h. The cells were then washed twice, resuspended in 2.8 ml FITC-conjugated horse-antirabbit F(ab')2 at 28 μg/ml, and incubated again for 1 h in the cold. After two further washes the cells were resuspended in 10 ml DAB/10% FCS/3 mM sodium azide. Surface IgM+ cells were separated from unlabeled cells using the FACS at University College of London. As a check on the specificity of labeling a smaller number of cells was similarly treated except that nonspecific rabbit F(ab')2 was substituted for the anti-IgM reagent used in the first incubation. Cell and reagent concentrations were identical in both procedures. To ensure that the lightly labeled IgM+ cells were included in the positive fraction the FACS threshold was chosen such that 40% of TDL were treated as positive (i.e., rather in excess of that obtained autoradiographically). The 10% of TDL immediately below this threshold were discarded and the remaining cells, 50% of the total, were selected as negative. Approximately 3% of cells were positively labeled by the control reagent.

Cells with surface IgG+, were selected by using rabbit F(ab')2-antirat IgG (Fd) at 14 μg/ml for the first incubation. Although the anti-IgG (Fd) reagent contained no detectable antibodies against rat IgM this incubation was carried out in the presence of purified rat IgM at 35 μg/ml. The FACS threshold was chosen so that 6% (exp. 1; Table IV) or 9% (exp. 2; Table IV) of TDL were selected as IgG+. The control reagent labeled 3% and 4.5% of TDL, respectively, in the two experiments. In both cases the 10% of cells immediately below threshold were discarded and the remaining cells were selected as negative.

Preparation of Helper T Cells. Carrier-primed T cells, required in adoptive transfer studies to supplement IgM+ or IgG+ cells obtained from the FACS, were obtained by depleting TDL from hapten-carrier-primed rats of IgL. Briefly, TDL were rosetted with SRBC coated with rabbit F(ab')2-antirat Fab and fractionated on a discontinuous gradient of Isopaque/Ficoll. Nonrosetting cells, which contained the helper T cells, were recovered from the upper layer of the gradient, washed twice in DAB/10% FCS, counted, and adjusted to the required concentration for injection. Details of the method are given elsewhere (5).

Results

The Class of Surface Immunoglobulin on CR+ and CR− Rat TDL. In order to determine the class of membrane immunoglobulin on CR+ and CR− TDL, the cells were labeled with 125I-rabbit F(ab')2-antirat immunoglobulin reagents, rosetted with SRBC-antibody-mouse complement complexes, smeared, and fixed. Autoradiographs were examined for rosettes and labeled cells, 1,000–2,000 cells being examined on each slide. The results, presented in Table I, showed that virtually all CR+ cells were Ig+, that the majority of Ig+ cells carried surface IgM, and that over 90% of the IgM+ cells were CR+. In contrast, only 2% of cells carried surface IgA and the majority of these lacked a receptor for C3. The absence of labeled rosettes on the control slide, prepared from lymphocytes preincubated, before rosetting, in medium only, established that stress artifacts (10) did not produce false labeling. The percentage of TDL that were CR+ on the autoradiographs (32–34%) was slightly less than that determined in a hemocytometer before smearing (35.6%), suggesting that a small percentage of rosettes were disrupted in the preparation of smears.

To avoid the necessity of smearing rosettes, TDL were labeled with 125I-rabbit F(ab')2-antirat immunoglobulin reagents after depleting TDL of CRL rosettes on an Isopaque/Ficoll gradient. Control TDL were treated similarly except that unsensitized SRBC were substituted for EAC in the rosetting procedure. In Table II the results of two such experiments are given. The findings were similar in both cases, confirming the existence of CR+ Ig+ and CR− Ig− subpopulations (5, 13) and indicating that few, if any, cells with surface IgM lacked C3 receptors. The enrichment of IgA+ cells produced by CRL depletion suggests, as
Table I
Surface Labeling of CR⁺ and CR⁻ Cells in Rat TDL

| Cell surface labeled with ¹²⁵I-rabbit F(ab')₂-antirat: | Percentage of cells | CR⁺ | CR⁻ |
|-----------------------------------------------------|---------------------|-----|-----|
|                                                     | Labeled | Unlabeled | Labeled | Unlabeled |
| Fab                                                 | 33.2    | 0.2       | 5.3     | 61.3     |
| IgM                                                 | 30.1    | 2.9       | 1.2     | 65.8     |
| IgA                                                 | 0.3     | 3.7       | 1.7     | 66.3     |
| Control                                             | 0       | 34        | 0       | 66       |

35.6% of TDL were CR⁺ as determined by examination of cell suspensions in a hemocytometer.

Table II
Surface Labelling of Normal and CRL-Depleted TDL

| Cell surface labeled with ¹²⁵I-rabbit F(ab')₂-antirat: | Exp. 1 | Exp. 2 |
|--------------------------------------------------------|-------|-------|
|                                                        | Undepleted | CRL depleted* | Undepleted | CRL depleted* |
| Fab                                                   | 33.4   | 4.0    | 36.0     | 5.5       |
| IgM                                                   | 31.6   | 0.3    | 29.6     | 0.2       |
| IgA                                                   | 1.9    | 3.5    | 2.4      | 3.1       |
| IgG                                                   | 3.0    | 0.6    | ND†      | ND†       |

* Results are expressed as a percentage of cells on the autoradiographs and not as a percentage of cells present before CRL depletion.
† ND, not determined.

was found in the double-labeling experiment (Table I), that the majority of IgA⁺ cells were in the CR⁻ subpopulation. Cells with surface IgG were about as abundant as those with surface IgA but, in contrast to the latter, the majority were CR⁺.

The Class of Surface Immunoglobulin on Rat TDL Mediating IgG Memory.
In the previous paper (5) it was reported that hapten-carrier-primed TDL transferred a response in which IgG PFC were approximately 100-fold more abundant than IgM PFC. In contrast, the results in Table II show that the great majority of Ig⁺ cells in TDL carry surface IgM. In order to determine the IgG precursor potential of both IgM⁺ and IgG⁺ cells in rat TDL it was necessary to employ the FACS because the IgG⁺ subpopulation was too small (~3% of TDL) to separate and test by other procedures.

Cells were obtained from the thoracic duct of rats primed 4-6 wk earlier with dinitrophenyl conjugate of bovine gamma globulin. Using appropriate fluorescent surface labeling, IgM⁺ or IgG⁺ cells were separated from unlabeled cells on the FACS. The positive and negative fractions, supplemented with helper T cells, were transferred together with antigen into irradiated syngeneic recipients. Plaque-forming cell assays were carried out 7 days later. In Table III the results of selecting IgM⁺ cells are presented. It is apparent that the IgM⁺ fraction contained the majority, if not all, of the IgG antibody-forming cell
### Table III

The Capacity of IgM⁺ and IgM⁻ Subpopulations of Rat TDL Supplemented with Carrier-Primed T Cells, to Transfer an Adoptive 7S Response

| TDL transferred                          | No. of recipients | Indirect PFC/spleen (mean and range) |
|------------------------------------------|-------------------|--------------------------------------|
| 7.4 × 10⁸ T cells*                       | 4                 | 427 (−64 − 220)                      |
| 7.4 × 10⁸ T cells + 6.0 × 10⁷ IgM⁺ cells§ | 2                 | 70,025 (56,700−84,350)               |
| 7.4 × 10⁸ T cells + 6.0 × 10⁷ IgM⁻ cells|| 2                 | 358,600 (269,200−448,000)            |
| 10⁷ Unseparated TDL                      | 4                 | 185,100 (154,800−221,700)            |

Recipient rats, syngeneic with the TDL donors, received 700 rads 24 h before cell transfer.

* TDL from primed donors depleted of IgL as described in Materials and Methods.

§ Negative results because there were fewer plaques in assay chambers containing developing serum than in those which were undeveloped. The responses were in all cases negligible compared with those in the other groups.

§ Contaminated with 5 × 10⁷ IgM⁻ cells.

|| Over 99% pure.

recipient rats, syngeneic with the TDL donors, received 700 rads 24 h before cell transfer.

precursors. When IgG⁺ cells were selected (Table IV) the reverse result was obtained, the positively labeled cells providing the great majority of the adoptive IgG response. Since, in these latter experiments, 10–20 times as many IgG⁻ cells as IgG⁺ cells were transferred the results imply that, compared with the IgG⁻ fraction, the IgG⁺ cells contained an approximately 100-fold higher concentration of IgG precursors.

In a supplementary experiment the FACS was used to verify the magnitudes of the IgM⁺ and IgG⁺ subpopulations in rat TDL. Using saturating concentrations of antibody to label the cells and identical machine operating conditions, 35% of cells labeled with the rabbit F(ab')₂-antirat IgM reagent while only 2.75% labeled with the corresponding antirat IgG (Fd) antibody.

### Discussion

TDL from primed rats conferred on syngeneic irradiated recipients the ability to generate a substantial IgG anti-DNP response (>2 × 10⁷ PFC/spleen) after challenge with the immunizing conjugate. Fractionation of the donor lymphocytes on the FACS showed that the ability to transfer IgG memory was associated with the small percentage of TDL (~3%) bearing surface IgG. Thus, both the CR⁺ and CR⁻ IgG memory cells described in the previous paper (5) carry this class of membrane immunoglobulin. This finding did not support the hypothesis, advanced in the introduction, that these two subpopulations represented different stages in the maturation of memory cells. The identity of class of membrane and secreted immunoglobulin has also been observed in studies on murine spleen cells (1, 14) and rabbit lymph node and Peyer's patches cells (15). More recently, however, (4) it has been suggested that memory cells in the mouse carry membrane IgD. Since IgD has not yet been identified in the rat it can only be said that if IgG memory cells in rat TDL carry IgD, then they do so in addition to IgG. Cross-reactivity of the anti-IgG₂ (Fd) reagent for IgD determinants is unlikely in view of the fact that the reagent failed to label IgM⁺ cells, some of which would be expected to carry both IgM and IgD (16). However,
TABLE IV

The Capacity of IgG⁺ and IgG⁻ Subpopulations of Rat TDL to Transfer an Adoptive 7S Response

| Exp. | TDL transferred | No. of recipients | Indirect PFC/spleen (mean and range) |
|------|----------------|-------------------|-------------------------------------|
| 1    | 6 × 10⁶ T cells | 1                 | 200                                 |
|      | 6 × 10⁶ T cells + 4.3 × 10⁶ IgG⁺ cells* | 3 | 88,175 (51,250–124,550) |
|      | 6 × 10⁶ T cells + 1.2 × 10⁷ IgG⁻ cells$ | 3 | 9,400 (8,300–10,500) |
|      | 10⁷ Unseparated TDL | 4 | 37,760 (18,300–71,350) |
| 2    | 6.6 × 10⁶ T cells | 4                 | 125 (−150 − 350) |
|      | 6.6 × 10⁶ T cells + 6.9 × 10⁷ IgG⁺ cells§ | 3 | 82,815 (69,650–100,750) |
|      | 6.6 × 10⁷ T cells + 8.7 × 10⁷ IgG⁻ cells|| | 3 | 9,115 (4,150 − 15,200) |
|      | 1.1 × 10⁷ Unseparated TDL | 4 | 140,425 (101,150 − 166,150) |

Recipient rats received 850 rads (first experiment) or 750 rads (second experiment) 24 h before cell transfer.

* Contaminated with 1.6 × 10⁶ IgG⁻ cells.
$ Over 97% pure.
§ Contaminated with 2.3 × 10⁶ IgG⁻ cells.
|| Over 99% pure.

The capacity of the reagent with IgG subclasses other than IgG₂ was not examined and it is conceivable that the CR⁺ and CR⁻ IgG memory cells are of a different subclass.

The result of comparing the percentage of cells bearing rat light chains with the sum of those bearing IgA, IgG, or IgM indicates that there can be few, if any, Ig⁺ cells in rat TDL carrying unidentified heavy chains. These findings are similar to those of Williams and his colleagues who first described the frequency of rat B lymphocytes bearing different Ig classes (6, 8, 11). The functional studies exclude the possibility that the IgG bearers also carry large amounts of surface IgM, and the same holds for cells with both internal and membrane IgA (11). It can be concluded that if an equivalent to human IgD (4, 16) exists in the rat then few cells in rat TDL bear such an immunoglobulin exclusively.

Although the few percent of TDL that carried surface IgG produced an adoptive IgG response that was nearly 10 times greater than that transferred by almost 10 times as many IgG⁻ cells, the IgG⁻ fraction did contain some IgG precursor cells. Similarly, a small response was obtained when IgM⁺ cells were transferred together with helper T cells. Whether these small responses arose from contaminating IgG⁺ cells or from precursor cells with membrane IgM is not known. It is notable that, when selecting IgG⁺ cells on the FACS, the adoptive IgG response obtained from the IgG⁻ fraction was unaffected by changing the percentage of cells selected as IgG⁺ from 6 to 9%, suggesting that contaminating IgG⁺ cells were probably not responsible for these small responses. There is evidence from several studies that some memory cells for IgG responses bear membrane IgM. Thus Pierce et al. (2) observed a reduction in magnitude of a secondary response in vitro when anti-μ-antibody was added to the culture and Jones et al. (15) concluded that some IgM⁺ cells differentiated into cells containing cytoplasmic IgA or IgG in cultures of rabbit lymphocytes. Press and Klin-
man (17), studying secondary immune responses in the mouse showed that approximately 16% of plasma cell clones synthesizing IgG antibody also produced IgM.

The class of immunoglobulin on IgM precursors in rat TDL was not firmly established. However, the finding that IgM precursors are CR+ (5), that virtually all cells with surface IgM are also CR+, and that all Ig+ cells can be accounted for by the sum of the cells possessing membrane IgA, IgG, or IgM, provides indirect evidence for the currently accepted view (3) that such cells are IgM+.

It has been suggested by Strober (18) that the majority of Ig+ cells in rat TDL are memory cells. The present observations do not appear to support this conclusion. Thus, although the IgM+ population comprised over 80% of all Ig+ cells in TDL such cells could have contributed, at most, about 10% of the adoptive IgG response. As previously reported (5) the adoptive IgM response was very small. However, it could be argued that the irradiated recipient does not provide a suitable environment for the expression of memory by IgM+ cells. Although this explanation cannot be excluded it is possible that the infrequency of precursors reactive to certain antigens in primary responses (19) may reflect a much wider repertoire of specificities among virgin, as opposed to memory B cells.

It is generally accepted (20) that CRL in the mouse are B cells but the presence of CR+ T cells in mouse spleen has also been reported (21). The observation, in the present study, that very few CR+ cells lacked surface Ig indicates that, in rat TDL, CR+ cells are bone marrow derived. The presence of the receptor for C3 on both IgM+ and IgG+ cells would appear to rule out the possibility that this receptor is involved in blocking a switch from IgM to IgG synthesis (22). The identification of CR+ and CR- IgG memory cells, both bearing membrane IgG, provides a useful system in which to study the function of this receptor.

Finally, it must be emphasized that rat TDL, although capable of mediating a wide range of immunological responses (23) are not representative of all rat lymphocytes. Thus, in studies on rat spleen (D. W. Mason, unpublished data) it has been found that although the great majority of Ig+ cells carry surface IgM only half of these cells possess C3 receptors; and Dukor et al. (22) and Parish (24) in studies on murine splenic lymphocytes have found IgM precursors in both CR+ and CR- subpopulations.

Outstanding problems raised by the present work are the origin of the IgG+ memory cells, the mechanisms by which they are generated during priming with antigen, and the function of the large recirculating pool of IgM+ cells.

**Summary**

The fluorescence activated cell sorter was used to determine the class of immunoglobulin on the thoracic duct lymphocytes (TDL) which carried IgG memory. Although only about 3% of all TDL carried membrane IgG these cells accounted for most, if not all, of the adoptive IgG anti-DNP response. It is concluded that both CR+ and CR- cells mediating IgG memory in rat TDL bear the same class of membrane immunoglobulin as that secreted by their differentiated progeny.
The class of membrane immunoglobulin on CR+ and CR− rat TDL was also examined. It was found that IgM+ cells, which made up over 80% of all Ig+ cells, were virtually all CR+. In contrast, the few percent of IgG+ and IgA+ cells present were to be found in both subpopulations. There was no evidence of a large population of B cells bearing exclusively heavy chains other than IgA, IgG, or IgM. The observation that some IgG+ cells as well as IgM+ cells possess a receptor for C3 appears to rule out the hypothesis that this receptor is involved in blocking a switch from IgM to IgG synthesis.

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