Long-term antibodies after an oral immunization with cholera toxin are synthesized in the bone marrow and may play a role in the regulation of memory B-cell maintenance at systemic and mucosal sites

R. Benedetti, P. Lev, E. Massouh and J. Fló (*)

Laboratory of Immunochemistry, Faculty of Exact and Natural Sciences, University of Buenos Aires, Buenos Aires

SUMMARY

To study the importance of the bone marrow in the long-term antibody response, IgG and IgA antitoxin antibody-forming cells were evaluated by ELISPOT in Peyer's patches, mesenteric lymph nodes, spleen, lamina propria of the small intestine and bone marrow at several times after oral immunization with cholera toxin. The mesenteric lymph node was the site having the major frequency of IgG antitoxin during the first two weeks after priming, whereas lamina propria was the site with a major number of IgA antitoxin antibody-forming cells. However, from 3 weeks until 10 months after priming, bone marrow became the site with the major frequency of IgG, and especially IgA antitoxin antibody-forming cells (without taking into account the lamina propria). This result indicates that bone marrow was responsible for the long-term antibody response and raises questions concerning the mechanisms involved in the maintenance of antibody production. The importance of bone marrow as a site of antibody production was great when we analysed results as the true contribution of the total number of antitoxin antibody-forming cells, taking into account the number of cells recovered from each organ. When we analysed the anatomical location of memory B and T cells by adoptive transference, we found that cells from mesenteric lymph nodes and spleen were able to transfer a strong antibody response to naive syngeneic recipients, whereas bone marrow cells transferred a weak antibody response.

Key-words: Bone marrow, Immunogenesis, B lymphocyte, Cholera toxin, IgA, IgG; Mucosal immunization, Long-term antibody response, Anamnestic response, Follicular dendritic cells, Rat.

INTRODUCTION

It is well described that Peyer's patches (PP) are the main site at which the mucosal immune response is induced. Antigens present in the gastrointestinal tract, once transported by M cells (which are associated with the epithelium covering the PP), could interact first with the antigen-presenting cells and lymphocytes present in the intraepithelial pocket of the...
M cells. In the dome region below the follicle-associated epithelium (FAE), IgM+ B cells, CD4+ T cells, dendritic cells and macrophages form a cellular meshwork in which the antigens are likely to be taken up, processed and presented to lymphocytes, which would then be activated (Emark and Owen, 1986). Following activation, B cells in PP undergo a process of maturation and differentiation. In PP, T lymphocytes, stromal cells and antigen-presenting cells produce specific lymphokines that regulate the differentiation of B lymphocytes and control their commitment to IgA synthesis (Cebra et al., 1976; Nedrud and Lamm, 1991).

Cholera toxin (CT) is an exception in that it is able to produce strong secretory immune responses after either oral or other mucosal routes of immunization both in experimental animals and humans (Pierce and Cray, 1981, 1982; Lycke et al., 1985; Lycke and Holmgren, 1986a,b). Furthermore, a second unusual property of CT is that of mucosal adjuvanticity; i.e., when a second, unrelated antigen is coadministered with CT in the intestine, a secretory IgA response to unrelated antigen is generated even though no such response occurs when the antigen is fed by itself (Lycke and Holmgren, 1986a,b; Elson, 1992; Lycke et al., 1992; Holmgren et al., 1994). There are older reports, however, showing that CT given intragastrically to mice resulted in a substantial serum antibody response to CT. This antibody response was mainly of the IgG class (Fujita and Finkelstein, 1972; Elson and Ealding, 1984). These results of feeding CT were puzzling, because the feeding of most other proteins in substantially larger amounts did not result in a significant serum antibody response.

It is well established in mammals that after systemic immunization, the bone marrow (BM) is the major site of antibody production in a secondary immune response (Benner and Haaijman, 1980; Benner et al., 1981). The induction of the immune response occurs in lymphoid tissues other than BM, and then the stimulated cells migrate from the secondary lymphoid organs to the BM (Kosco et al., 1988; DiLosa et al., 1991).

However, until now there has been little information about the role of BM in antibody production after mucosal immunization. Previously, we had shown in rats that three weeks after one oral dose of CT, the BM was the main site in the production of circulating antitoxin antibodies, mainly of the IgA isotype (Benedetti et al., 1995). This result links the BM with the mucosal immune system. One point that remains to be addressed is the role of the BM in the persistence of long-term antibodies. The presence of circulating antiviral antibodies over the long-term after a viral infection is extensively described. These pre-existing antibodies are an important defence mechanism against reinfection (Ahmed, 1992; Gray, 1993). However, there is little evidence concerning the long-term antibody immune response for antigens administered by a mucosal route. Many questions concerning the mechanisms involved in these responses, as well as the nature and anatomical location of antibody-forming cells (AFCs), remain to be clarified.

In the present study, we sought to determine the anatomical site of long-term antibody production after oral priming with CT, as well as the role of these antibodies. Furthermore, we studied the role of the BM in the location of B and T memory cells.

| AFC         | antibody-forming cell. |
| BM          | bone marrow.            |
| CT          | cholera toxin.          |
| ELISPOT     | ELISA spot assay.       |
| FAE         | follicle-associated epithelium. |
| FCS         | foetal calf serum.      |
| FDC         | follicular dendritic cell. |
| GALT        | gut-associated lymphoid tissue. |
| i.v.        | intravenous(ly).        |
| LP          | lamina propria.         |
| mAb         | monoclonal antibody.    |
| M           | microfold (cells).      |
| MLN         | mesenteric lymph node.  |
| PBS         | phosphate-buffered saline. |
| PNA         | peanut agglutinin.      |
| PP          | Peyer's patch.          |
MATERIALS AND METHODS

Animals, immunizations and sample collection

Female syngeneic Wistar rats (WKAH/Hok) 8-12 weeks of age were used. Rats were maintained under constant environmental conditions on commercial rodent chow and water ad libitum throughout the study. Rats were immunized intragastrically as follows. Three weekly oral doses of 20 μg of CT (Sigma Chemical Co., St. Louis, MO) dissolved in 3 % sodium bicarbonate were administered by means of a neonate cannula under light anesthesia. At different times, rats were killed and samples collected. Rats were bled under ether anesthesia and sera stored at -20°C. Intestinal secretions were obtained as described previously (Fió et al., 1994a,b). To study the anamnestic immune response, ten months after the priming, rats were challenged with one oral dose of 20 μg of CT. Two, three and five days after the booster, rats were killed and samples were collected as described above.

Preparation of lymphoid cells

Single-cell suspensions of PPs, mesenteric lymph nodes (MLNs), spleen and BM were prepared as described previously (Benedetti et al., 1995; Fió et al., 1994a,b). Approximately 95 % of the recovered lymphoid cells were viable, as revealed by trypan-blue exclusion.

Single-cell suspensions were prepared from the intestinal lamina propria (LP) as follows. Segments of the small intestine were rinsed in cold PBS pH 7.3 and the PPs were excised; after opening along the mesenteric border, the strips were washed vigorously in citrate buffer pH 7.2. They were then incubated for 3 min with rotation in 1 mM dithiothreitol in citrate buffer, washed twice in citrate buffer, then placed in citrate buffer containing 2 % bovine serum albumin at 37°C and rotated for a further 15 min followed by vigorous vortexing for 30 s to dislodge the epithelium. The remaining gut segments were then chopped and passed through a wire mesh sieve into citrate buffer, then eluted through cotton wool columns, centrifuged and washed in RPMI containing 5 % foetal calf serum (FCS). Approximately 50 % of the recovered lymphoid cells were viable, as revealed by trypan-blue exclusion.

Adoptive transfer of memory

Memory cells were transferred 10 months after priming immunizations to naive recipient syngeneic rats. The cells were isolated from MLNs, spleen or BM as described above and injected intravenously into the lateral tail veins of the recipient animals. The transferred cells from MLNs and spleen did not contain any antitoxin-secreting cells, and cells from BM contained less than 1:10⁶ antitoxin-secreting cells, as tested with the ELISPOT technique. The mice were then challenged perorally with 20 μg of CT two days after cell transfer (days 2 and 4). Control animals received cells from non-immunized rats and were then orally challenged with CT. Serum and intestinal lavage were collected and cells were isolated from MLNs, spleen and BM after 6 or 7 days following the transfer of memory cells.

Depletion of T and B cells prior to transfer

T cells (non-B cells) were prepared from whole spleen or BM cells by negative panning. Petri plates (15x100 mm, Falcon Labware, Oxnard, CA) were precoated with goat F(ab')₂, anti-mouse IgG (25 μg/ml) in carbonate-bicarbonate buffer pH 9.5 overnight at 4°C. The plates were then washed 3 times with PBS and blocked with complete medium (10 % FCS) for 30 min at room temperature. MLN, spleen or BM cells were incubated with an appropriate dilution of the pan-B mAb OX33 (anti-CD45RA) at 4°C for 30 min. After three washes with complete medium, 5x10⁷ cells were resuspended in 4 ml of medium and were added to each plate. Then the plates were incubated for 90 min at 4°C. The non-adherent cells were gently collected in order to avoid removal of T cells bound to the solid phase. This procedure was repeated twice and non-adherent cells served as T-cell-enriched fractions. When these cell fractions were analysed by flow cytometry, <1 % of cells were OX33⁺ (CD45RA⁺). B cells (non-T cells) were prepared as described above and the anti-CD3 mAb G4.18 was used. The analysis of the non-adherent fractions resulted in <2% CD3⁺.

Determination of antitoxin antibody levels

Antitoxin antibodies were determined by enzyme-linked immunosorbent assay (ELISA) as described previously (Fió et al., 1994). For specific IgA, intestinal fluid samples were analysed at dilutions of 1:5 and 1:50. Sera were analysed at dilutions of 1:100 and 1:1,000. Antitoxin levels in sera and in intestinal fluid were expressed as arbitrary ELISA units obtained from standard curves derived from 40 samples with different levels of anti-CT antibodies with a cutoff of 0.2 above the background. Every time, a reference sample was simultaneously run with the unknown samples.
Enumeration of anti-CT AFCs by ELISPOT

CT-specific AFCs were quantified using an ELISA spot assay (ELISPOT) as described previously (Benedetti et al., 1995). Briefly, the lids from polystyrene plates (Coming Glass Works, Coming, NY) were coated with GM1 ganglioside (Sigma) at a concentration of 6 µg/ml in PBS and incubated overnight at room temperature. After rinsing three times with PBS, purified CT (6 µg/ml in PBS) was added and the lids were incubated as described above. Residual protein-binding sites were blocked with complete medium for 2 h. After the medium was removed, increasing numbers of test cells in complete medium were added and cultured at 37°C in a CO₂ incubator for 6 h. The cells were removed by three washes with PBS containing 0.05 % Tween-20 and two washes of H₂O-Tween. The plates were then incubated overnight at 4°C with affinity-purified goat anti-rat α (Bethyl), γ (Sigma) or µ chain (Organon Tecknika Co., Cappel Division) antisera. Then the plates were washed and treated with IgG affinity-purified rabbit anti-goat IgG/horseradish peroxidase conjugate (Zymed Laboratories Inc., San Francisco, CA) for 2 h. Antigen-specific spots were developed in agar substrate containing 1% Noble agar (Difco), 0.5 mg/ml p-phenylenediamine (Sigma) and 0.1% H₂O₂. Spots corresponding to the zones of antibodies secreted by individual cells were enumerated under low magnification (×12), and data were adjusted to numbers of spot-forming cells per 10⁷ nucleated cells. The dilutions yielding between 20 and 100 spots/well were used to estimate the AFC frequencies shown.

To calculate the number of AFCs per organ, we made use of the results of other authors (Deenen et al., 1987), who demonstrated with ⁵⁹Fe-labelling that one femur of a rat contains 9% of its total bone marrow. Although the distribution of ⁵⁹Fe is not necessarily representative of the distribution of lymphocytes, it gives a clue as to the total amount of bone marrow.

Flow cytometry of BM cells

Cell suspensions from BM of one-year-old rats were prepared as described above. Pretitrated unconjugated mAb was added to 10⁶ cells at 4°C and incubated for 30 min. After a primary incubation, samples were washed three times in PBS at 4°C. The cells were resuspended, as noted above, and pretitrated F(ab')₂ goat anti-mouse IgG, FITC-conjugated (not cross-reactive with rat) (Cappel), were added as appropriate. The secondary reaction was then incubated and washed as described previously. After mAb staining and washing, all samples were fixed with 1% paraformaldehyde in PBS and stored at 4°C before FACScan analysis. The unconjugated pan-B primary mAb used was the OX33 (CD45RA) and the pan-T was the G4.18 (CD3). The samples were run on a FACScan flow cytometer (Becton Dickinson). Data were collected for 10,000 events. Isotype-matched antibodies were used as negative controls.

For BM cell analysis, the gate was set on the small lymphoid fraction by forward angle and 90° light scatter.

Statistical analysis

Statistical significances were determined by the Mann-Whitney U test.

RESULTS

BM contained the highest number of antitoxin AFCs after priming with three oral doses of CT

When we analysed the kinetics of the antibody immune response in serum, we found that the IgG antitoxin antibodies persisted even at 10 months after priming. However, 6 months after immunization, no IgA antitoxin antibodies were found in intestinal lavage. To determine the origin of these antibodies, primed rats were monitored at various intervals by collecting PPs, MLNs, lamina propria, spleen and bone marrow samples and screening for the presence of IgG and IgA antitoxin AFCs with the ELISPOT assay. For the first three weeks, MLN was the site with the major frequency of IgG antitoxin AFCs. Two months after priming, BM became the site with a major frequency of antitoxin AFCs. Beyond three months after priming, BM was the only site with significant numbers of IgG antitoxin AFCs (fig. 1a). When the frequency of IgA antitoxin AFCs was analysed, the LP was, as expected, the major site of IgA antitoxin production in the first 5 months. When the systemic organs were analysed, BM was the major site of IgA antitoxin production early in the immune response, and from five months after priming to the end of the experiment, it was the only site with a significant number of IgA antitoxin AFCs (fig. 1b).

To define the relative contribution of the different organs to total specific AFCs, their total
antitoxin plasma cell populations were compared. As shown in figures 1c and 1d, BM was by far the main site at which antitoxin IgA, and, to a lesser extent, antitoxin IgG, were produced throughout the study. As we do not know the total number of lymphoid cells in the LP, the total antitoxin AFC was not estimated in this site.

The frequency of antitoxin plasma cells in BM increased dramatically if we expressed the results as AFC per mature lymphoid cells, since in BM only 10% of the nucleated cells were mature lymphocytes, as we observed by flow cytometry.

To analyse whether preferential localization of the IgA antitoxin AFC occurred in BM in comparison to other tissues (excluding the LP), the ratio of IgA antitoxin per IgG antitoxin AFC was analysed in each organ. Throughout the kinetics, BM showed a significantly higher ratio of IgA anti-CT per IgG anti-CT AFCs than the MLNs or the spleen (fig. 2). Furthermore, in all tissues studied, this ratio increased with time.

**Bone marrow was the main site of antibody production in the early anamnestic antibody response after oral challenge with CT**

Two days after the booster, no differences were observed in the level of IgG antitoxin antibodies in serum when compared with the controls (without booster), but 3 days after the booster, a substantial increase was observed (1,960 ± 380
The IgA/IgG ratio was monitored several times after priming with three oral doses of CT. The Mann-Whitney U test was used. (*) P<0.01 and (**) P<0.05 when BM vs. spleen was compared.

IgG ELISA units). Five days after the booster, a dramatic increase in the levels of antitoxin IgG was observed (24,000±5,000 IgG ELISA units). Similar results were found when the antitoxin IgA was monitored in intestinal lavages (50±30 ELISA units at day 3 and 900±400 ELISA units at day 5 postbooster). No antitoxin activity was observed in control rats that received only one dose of CT, and the levels of antitoxin IgG and IgA were monitored in serum and in intestinal lavage, respectively, 2, 3 and 5 days after immunization.

To define the role of different organs in the production of antitoxin antibodies in the early anamnestic antibody immune response, rats were challenged 10 months after priming. Two, three and five days after the booster PPs, MLNs, LP, spleen and BM were screened for the presence of antitoxin AFCs. Two days after the booster, no differences were observed in the number of antitoxin AFCs between control rats (primed only) and challenged rats. Three days after the booster, MLNs had the major frequency of antitoxin AFCs, and the response in BM was similar to that observed in the spleen. Five days after the booster, a sharp increase in the number of specific IgG and IgA AFCs was observed in LP, BM and spleen (fig. 3a and 3b). At that time, very weak antitoxin activity was observed in the spleens of rats that were not primed, and that received one dose of CT. As shown in figures 3c and 3d, when the results were expressed as antitoxin AFCs per organ, BM became the site with the major contribution to the total specific AFCs in the early anamnestic antibody response.

**BM had memory antitoxin cells, but not in a significant number**

Single-cell suspensions of MLN, spleen or BM were prepared from rats that had been primed by three oral doses of CT 10 months prior to the experiment. Different doses of cells were injected i.v. into syngeneic rats. After 2 and 4 days, recipient rats were orally challenged with CT, and at day 6 or 7 after transfer, the animals were sacrificed and analysed for antitoxin antibodies in serum and intestinal lavage and for antitoxin AFCs in different tissues. A strong anamnestic antitoxin response was elicited by oral challenge with antigen when MLNs or spleen cells were transferred. A clear dose dependence was evident in the antitoxin response. After transfer of $20 \times 10^7$ cells from spleen, there was a strong antitoxin antibody response in serum and in intestinal lavage, which decreased with the lower number of transferred cells (table I). When cells from MLN were transferred, the anamnestic antibody response in serum and intestinal lavage, after challenge, was stronger than when spleen cells were used. In rats which had received BM cells, the antitoxin response was much lower than in rats receiving MLN or spleen cells. To obtain an antitoxin response similar to that occurring when spleen cells were transferred, 4-fold numbers of BM cells were necessary. Also, a dose-dependent response was observed for BM cells (table I). When the antibody response was studied at the single-cell level, we found IgG and IgA antitoxin
Fig. 3. Oral challenge of rats with CT resulting in a rapid increase in the frequency of antitoxin AFCs in MLNs and BM (A and B).

Challenge with one oral dose of CT showed that the BM was the site of the major number of antitoxin AFCs per organ, especially IgA antitoxin AFCs (C and D).

Rats were primed with three oral doses of CT and ten months later were challenged with one oral dose of CT. Two, three and five days after the booster, the frequency of antitoxin AFCs was monitored by the ELISPOT assay. Two days after the booster, no differences were observed between control rats (primed only) and challenged rats in the number of antitoxins. Four rats were used for each point.

AFCs in MLNs, spleen and BM (table I). The number of antitoxin AFCs was higher in MLNs and BM than in spleen when MLNs or spleen cells were transferred. When BM cells were transferred, MLNs were the site with the highest number of antitoxin AFCs. The isotype distribution of antitoxin AFCs elicited in different tissues after oral challenge with CT in rats to which spleen or MLN memory cells had been transferred was similar to that observed in the early anamnestic immune response described above. BM was the site with the highest ratio of IgA/IgG antitoxin AFCs. However, when BM cells were transferred, MLNs had a high ratio of IgA/IgG antitoxin AFCs.

Long-term memory residing in B and T cells

Non-B cell (T cell) and non-T cell (B cell) suspensions were prepared by negative panning from rats immunized 11 months earlier. T and B cells were transferred by i.v. injection and the recipient rats were then orally challenged with CT. Similar antitoxin AFC numbers and antitoxin antibodies in serum or in intestinal lavage resulted after transfer of T or B cells from spleen (table II). However, the adoptive anamnestic antibody response elicited after challenge was lower than when the whole fraction of cells was transferred. When BM cells were transferred, both non-B and non-T cells
Table I. Adoptive transfer of antitoxin memory after oral priming immunizations with CT, by cells from MLNs, spleen or BM: influence of transferred cell number on activation of an adoptive memory for antitoxin response in various lymphoid tissues.

Antitoxin AFC/10^7 cells in:

<table>
<thead>
<tr>
<th>Origin of cells</th>
<th>No. of cells (10^7)</th>
<th>MLNs IgG</th>
<th>MLNs IgA</th>
<th>Spleen IgG</th>
<th>Spleen IgA</th>
<th>BM IgG</th>
<th>BM IgA</th>
<th>Antitoxin increase in serum</th>
<th>Antitoxin increase in intestinal lavage</th>
</tr>
</thead>
<tbody>
<tr>
<td>MLNs</td>
<td>5</td>
<td>142±74</td>
<td>87±26</td>
<td>98±17</td>
<td>58±11</td>
<td>315±11</td>
<td>267±28</td>
<td>20</td>
<td>9.5</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>181±73</td>
<td>112±35</td>
<td>96±14</td>
<td>67±10</td>
<td>147±35</td>
<td>102±25</td>
<td>4.2</td>
<td>3.4</td>
</tr>
<tr>
<td>Spleen</td>
<td>10</td>
<td>845±576</td>
<td>565±372</td>
<td>310±160</td>
<td>188±77</td>
<td>786±136</td>
<td>778±341</td>
<td>87</td>
<td>47</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>36±23</td>
<td>24±13</td>
<td>33±21</td>
<td>28±22</td>
<td>34±20</td>
<td>24±15</td>
<td>1.8</td>
<td>0.73</td>
</tr>
<tr>
<td>BM</td>
<td>10</td>
<td>75±19</td>
<td>108±39</td>
<td>46±12</td>
<td>28±6</td>
<td>48±20</td>
<td>42±4</td>
<td>11</td>
<td>3.7</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td>12±15</td>
<td>11±8</td>
<td>5±4</td>
<td>10±8</td>
<td>9±5</td>
<td>9±7</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

Different numbers of MLN, spleen or BM cells from rats orally primed with CT 10 months prior to experimentation were transferred to syngeneic recipient rats which were challenged with CT on days 2 and 3.

Mean value±standard error of the mean.

Table II. Adoptive transfer of antitoxin memory after oral priming immunizations with CT by B and T cells from spleen and BM.

Antitoxin AFC/10^7 cells in:

<table>
<thead>
<tr>
<th>Origin of cells</th>
<th>No. of cells (10^7)</th>
<th>MLNs IgG</th>
<th>MLNs IgA</th>
<th>Spleen IgG</th>
<th>Spleen IgA</th>
<th>BM IgG</th>
<th>BM IgA</th>
<th>Antitoxin increase in serum</th>
<th>Antitoxin increase in intestinal lavage</th>
</tr>
</thead>
<tbody>
<tr>
<td>SP B cells</td>
<td>4</td>
<td>119±42</td>
<td>28±8</td>
<td>42±4</td>
<td>48±26</td>
<td>27±2</td>
<td>30±6</td>
<td>3.6</td>
<td>5.9</td>
</tr>
<tr>
<td>SP T cells</td>
<td>4</td>
<td>164±50</td>
<td>34±7</td>
<td>39±16</td>
<td>23±17</td>
<td>69±51</td>
<td>57±51</td>
<td>4.3</td>
<td>8.8</td>
</tr>
<tr>
<td>BM non-T cells</td>
<td>15</td>
<td>58±36</td>
<td>16±16</td>
<td>36±18</td>
<td>52±4</td>
<td>29±3</td>
<td>34±7</td>
<td>2.1</td>
<td>3.3</td>
</tr>
<tr>
<td>BM non-B cells</td>
<td>15</td>
<td>44±40</td>
<td>18±12</td>
<td>20±8</td>
<td>24±22</td>
<td>38±11</td>
<td>26±9</td>
<td>2.1</td>
<td>3.1</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td>16±15</td>
<td>16±15</td>
<td>10±4</td>
<td>17±8</td>
<td>9±5</td>
<td>8.6±7</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

Spleen and BM cells from rats orally primed with CT 10 months prior to experimentation were transferred to syngeneic recipient rats which were challenged with CT on days 2 and 3. B and T cells were prepared by negative panning. Since the lymphoid fraction of small lymphocytes in BM was less than 20%, the cell fractions after panning were referred to as non-B or non-T cells.

Mean value±standard error of the mean.
LONG-TERM ANTIBODIES AFTER ORAL IMMUNIZATION

were able to transfer a weak anamnestic antibody response (table II).

DISCUSSION

In the present study, we found that BM was the main site of early long-term antibody production after oral immunization with CT. Even one year after immunization, we were able to detect antitoxin AFC in BM, but not in the spleen, MLNs or LP. Indeed, when one takes into consideration the heterogeneity of the BM cell population and normalizes the results to AFC/10⁷ lymphoid cells, on the basis that 90% of splenic cells and 10% of BM cells are mature lymphoid cells, the antibody response in the BM is considerable. Furthermore, when the number of anti-CT AFCs was considered per organ, BM was by far the site with the highest number of anti-CT AFCs (without taking into consideration the LP).

An interesting point was that the ratio of anti-CT IgA/IgG AFCs increased consistently with time in all lymphoid tissues studied. This suggests that the IgG AFCs migrated to the systemic organs faster than the IgA AFCs. Furthermore, throughout the period studied, BM had the highest ratio of IgA/IgG AFCs (without taking into consideration the LP). This indicates that after mucosal immunization, the IgA blasts induced in the gut-associated lymphoid tissue (GALT) preferentially seed the BM rather than the spleen. It was reported that BM cells produce a chemoattractant which may help locally to attract stimulated blasts into the BM (Tew et al., 1992). Our results support the idea that IgA and IgG blasts have a differing capacity to be attracted by BM cells.

It has been described in humans that lymphocytes from several lymphoid tissues have the capacity to produce both monomeric and polymeric IgA, and about 20-30% of the IgA-secreting cells in the BM were engaged in the synthesis of polymeric IgA (Tarkowski et al., 1991). It is well established that rat hepatocytes, unlike those of humans, express a secretory component, and so IgA can be removed from the serum to the intestine through the liver (Lemaitre-Coelho et al., 1978; Jackson et al., 1992). By this mechanism, BM could help to maintain specific IgA in the intestine during long periods of time. These results seem to indicate that BM belongs to the systemic as well as to the mucosal immune system as an effector site.

It was reported that after systemic immunization, specific blasts can be found as PNA⁵⁰ blast cells in the thoracic duct, lymph and blood about 3 days after secondary challenge (Kosko et al., 1989). Furthermore, it was shown that after a viral reinfection with lymphocytic choriome ningitis virus, the secondary reinfection produced a rapid induction of AFCs in the spleen followed by a delayed increase in AFC numbers in the BM (Slifka et al., 1995). In the present study, we found, in rats primed and boostered with CT by the oral route, that 3 days after the challenge BM was the site with the major number of antitoxin AFCs per organ. This could indicate that in our model, stimulated cells emigrated from GALT to BM faster than from systemic lymph nodes.

It has been suggested that BM may be a reservoir for memory IgA B cells that ultimately reenter the circulation and populate mucosal sites (Alley et al., 1986). This hypothesis has been supported by the fact that the great majority of all IgG- and IgA-secreting cells are localized in the BM and that they are deficient in germ-free mice, indicating that the IgG and IgA produced are predominantly directed against environmental antigens (Benner et al., 1981). Furthermore, the PPs of mature young mice have significant numbers of B cells containing cytoplasmic Ig, whereas aged mice have involuted PPs and most Ig-containing cells are found in the BM (Haaijman et al., 1977; Haaijman and Hijmans, 1978). A similar involution of PPs with age was observed in humans (Cornes, 1965).

The results presented herein show that long-term immunological memory for CT after an oral immunization with CT is carried by B and T lymphocytes. These results are different from those of Lycke and Holmgren (1989) who demonstrated in mice that the immunological memory is carried by B cells. It is not clear what the causes
are for this discrepancy: probably the fact that the species used as well as the protocol of immunization were different.

In rats which had received memory cells, irrespective of whether they were isolated from the MLNs or the spleen, oral challenge with antigen resulted in antitoxin AFC in MLNs, spleen and BM, as well as in high levels of specific IgA in the intestine, and the magnitude of the response was similar to that observed 5 days after a booster of optimally primed rats. When BM cells were transferred to naive recipient animals, a 5-fold number of cells was necessary to obtain a similar immune response to that when MLN or SP cells were used. If the number of spleen or BM cells transferred are normalized on the basis of the lymphoid fraction (10% in BM and 90% in spleen), spleen and BM seem to have similar numbers of memory cells, but on an absolute basis, BM seems to be less important in the contribution to the pool of memory cells than spleen. However, the importance of BM in the early anamnestic antibody response was confirmed, since six days after the challenge BM was the site with the highest number of antitoxin AFCs in the recipient rats.

It is well established that serum Igs have a short half-life, usually on the order of several days to, at most, a few weeks (Fahey and Sell, 1965). Thus, to maintain high levels of circulating antitoxin antibody, there must be a constant pool of plasma cells synthesizing antibody. The long-term maintenance of specific serum antibody levels in secondary immune responses has been attributed to an antibody feedback system in which antigen persisting on follicular dendritic cells (FDCs) plays an important role (Donaldson et al., 1986). It was reported that FDCs are able to retain antigen for years, and that antigen-bearing FDCs do not circulate and are restricted to organs draining the site of immunization (Tew et al., 1990). However, although all the immune cells necessary to mount an antibody response are present in BM, it is well described that no antigen presentation occurs in BM. Furthermore, our results obtained after severe bleeding suggest that after an oral immunization with CT, the antigen persists, probably on FDCs, in the MLNs but not in the BM (data not shown).

This brings up the interesting issue of plasma cell longevity. The current dogma is that plasma cells have a rapid turnover rate and are replenished by cycling B cells that differentiate within germinal centres of the spleen and lymph nodes (Nieuwenhuis and Opstelten, 1984; Maclennan et al., 1992). BM was described as containing factors which are capable of stimulating germinal centre B cells to produce high levels of IgG (Tew et al., 1992). In our experiments, the spots produced by BM AFC in the ELISpot assay were consistently larger in diameter than those produced by MLN or spleen AFC. It is possible to envisage that BM cells secrete soluble factors that not only enhance Ig synthesis, but also increase the life span of plasma cells.

The levels of the long-term antibodies could be related not only to prevention of infections, but also to maintenance of immunological memory (Gray, 1994). It is widely believed that FDCs bearing the antigen are necessary to support memory B cells. When the level of antibodies decreases, the immune complexes on FDCs are dissociated and the antigen is presented by B cells to T cells. Then, new AFC and memory B cells are generated in two different pathways, and the levels of circulating antibodies are reestablished. The results obtained by us suggest that MLN is the site at which the antigen persists 7 months after oral priming with CT (data not shown). MLNs receive lymphocyte draining both from the blood and from the PPs through the lymph. Furthermore, it is well established that cells leaving the MLN seek the LP as well as systemic organs. It is thus possible to hypothesize as to a link between long-term circulating antibodies and the maintenance of immunological memory in the mucosal tissues. Cells activated in MLNs after the decrease in the levels of circulating antibodies could migrate to the mucosal tissues (LP and probably PPs) as AFCs and as memory cells, and in this way, long-term memory could be maintained in the mucosal compartment.
LONG-TERM ANTIBODIES AFTER ORAL IMMUNIZATION

Acknowledgements

This work has been supported by grants from University of Buenos Aires (Ex 287).

The authors thank Mr Guillermo Assad Ferek for skilful assistance.

Persistant des anticorps, après immunisation orale avec de la cholérratoxine, synthétisées dans la moelle et pouvant jouer un rôle dans le maintien de la mémoire cellulaire B au niveau général et muqueux

Les cellules formatrices d'IgG et d'IgA antidoteine ont été étudiées (technique ELISPOT) dans les plaques de Peyer, les ganglions mésentériques, la rate, la lamina propria du grêle et la moelle osseuse après immunisation avec la cholérratoxine. Le ganglion mésentérique est le site le plus producteur d'IgG dans les deux premières semaines alors que la lamina propria est le site formant le plus de cellules productrices d'IgA. Dans les 3 semaines à 10 mois, la moelle devient le site principal de la formation d'IgG et d'IgA notamment (abstraction faite de la lamina propria). Ces résultats montrent que la moelle est responsable de la réponse anticorps de longue durée, ce qui soulève des questions concernant les mécanismes impliqués dans le maintien de la production des anticorps. L'importance de la moelle comme site de production est confirmée par les résultats montrant le rôle déterminant des cellules formatrices anticorps anti-toxine compte tenu du nombre des cellules de chaque organes. L'analyse du lieu anatomique des cellules B et T de la mémoire immunitaire au moyen du transfert adoptif révèle que les cellules des ganglions mésentériques et de la rate sont capables de transférer une forte réponse anticorps alors que les cellules de la moelle ne transfèrent qu'une réponse limitée.

Mots-clés : Moelle osseuse, Immunogenèse, Lymphocyte B, Cholérratoxine, IgA, IgG; Rat, Immunisation par les muqueuses, Réponse anticorps de longue durée, Réponse anamnestique, Cellules folliculaires dendritiques.

References


Lycke, N. & Holmgren, J. (1986b), Strong adjuvant properties of cholera toxin on gut mucosal immune responses to orally presented antigens. *Immunology*, 59, 301-308.


