LOCALIZATION OF IMMUNOLOGICAL COMPLEXES FIXING $\beta_{\text{M}}$ (C3) IN GERMINAL CENTERS OF LYMPH NODES*

BY K. J. GAJL-PECZALSKA,‡ M.D., A. J. FISH,§ M.D., H. J. MEUWISSEN,¶ M.D., D. FROMMEL,† M.D., AND R. A. GOOD,** M.D.

(From the Pediatric Research Laboratories of the Variety Club Heart Hospital, University of Minnesota, Minneapolis, Minnesota 55455)

(Received for publication 30 June 1969)

Germinal centers of lymphoid follicles, first described in 1885 as sites of proliferation of lymphocytes, have been intensively studied over the past 15 years (14, 75). Although it has been generally accepted that germinal centers play an important role in immunological defense, their precise function is not well known.

Antigen localization within germinal centers after primary and secondary immunization has been shown by many authors, using immunofluorescent and radioautographic techniques (1, 2, 17, 38, 42, 51, 56). These and more recent studies, employing electron microscopy (52), have suggested that localization of antigen in germinal centers occurs on the surface of dendritic processes of reticulum cells. These dendritic processes in turn are in intimate contact with lymphocytes and blastoid cells (52). The lymphocytes in germinal centers, many of which are actively replicating cells, have been regarded as precursors of plasma cells (21, 34–37, 80), or possible “memory cells” (12, 73, 76, 77).

Other authors have described the presence and production of immunoglobulins and antibodies in germinal centers (8, 15, 16, 45, 49, 59, 80, 81). However, Pernis has submitted evidence that at least some of the immunoglobulins present in germinal centers are not synthesized locally (61).

Germinal centers have been associated with antibody and immunoglobulin production in other ways. Germinal centers and plasma cells are regularly absent from lymph nodes, spleen, appendix, and tonsils of patients with the Bruton-type sex-linked form of hypogammaglobulinemia (28, 30, 33). When their development in the

* These investigations were supported by grants from the American Heart Association, The National Foundation–March of Dimes, and the U.S. Public Health Service (AI-08677 and NB-02042).
‡ Postdoctoral Fellow of the U.S. Public Health Service (international fellowship Fo5-TW01270).
§ Established Investigator of the American Heart Association.
¶ Career Development Awardee of the U.S. Public Health Service.
† Postdoctoral Fellow of the U.S. Public Health Service (cardiovascular training grant HE-05222).
** American Legion Memorial Heart Research Professor of Pediatrics and Microbiology.
chicken is experimentally prevented by sublethal irradiation and with extirpation of the bursa of Fabricius at hatching, 19S and 7S immunoglobulin synthesis and antibody formation are ablated (9, 19). Similarly, extirpation of the bursa in ovo alone, when carried out sufficiently early in development (e.g. after 15-17 days of incubation), will prevent IgG and IgM synthesis and destroy the ability to form plasma cells and germinal centers (9). Finally, bursectomy alone in 18 or 19 day old chick embryos prevents germinal center formation and 7S IgG synthesis, although plasma cell development and IgM production are not impaired (18).

Similar distribution of antigen and antibody in germinal centers was shown using radioautography and immunofluorescence techniques (4, 47, 82). Complement or complement components, however, have never been shown to be present within germinal centers. Indeed, Pernis and collaborators have presented data indicating that $\beta_{1c}$-globulin component of complement (54) is regularly absent from germinal centers even though immunoglobulins and antigen are demonstrable (62). They concluded from their observations that antigen-antibody complexes present in germinal centers are, for reasons as yet poorly understood, unable to bind complement in vivo.

It was the purpose of our study to analyze this question further. We present data which show that antigen, immunoglobulins (IgG and IgM), antibody, and $\beta_{1c}$ are localized within germinal centers, and that all these components have a similar distribution within these sites. It appears likely that IgG, IgM, and $\beta_{1c}$ are not synthesized by cells in the germinal centers, but are deposited there as antigen-antibody complexes with $\beta_{1c}$. We have achieved complete elution of IgG, IgM, $\beta_{1c}$, and antigen (bovine serum albumin, BSA) from the germinal centers in the rabbit. Furthermore, we have demonstrated the capacity of the complexes in germinal centers of the rabbit to fix heterologous (human) complement. These data present strong evidence that antigen-antibody complexes within germinal centers fix $\beta_{1c}$ component of complement in vivo. There is indication that antigen-antibody complexes in germinal centers are located on the surface of cells, presumably dendritic macrophages; from this extracellular site complexes can be readily removed by standard elution procedures.

Materials and Methods

Patients.—28 lymph nodes from 26 patients on the pediatric, surgical, and medical services of the University of Minnesota Hospitals, obtained at surgery or at autopsy (no later than 6 hr after death), were investigated. The diagnoses of the patients from whom lymph nodes were obtained differed widely, but the gross appearance and general histological architecture of the lymph nodes used in this study were normal and revealed no evidence of malignant infiltration or pathological distortion.

Animals.—60 New Zealand white rabbits weighing approximately 5-6 lb. were obtained from a single breeder, housed in metal cages, and provided with tap water and Purina rabbit pellets ad libitum. The rabbits were divided into three groups treated as follows.

Group I: Rabbits immunized with Brucella antigen; A. Popliteal lymph node stimulation: 12 rabbits were immunized initially with Brucella antigen (U.S. Department of Agriculture, Washington, D.C.); $2 \times 10^{10}$ killed Brucella cells (0.1 ml) were mixed in an equal volume of complete Freund's adjuvant and injected subcutaneously into a single foot pad. 3 wk later
0.1 ml containing \(2 \times 10^{10}\) Brucella organisms without adjuvant was injected into the same foot pad in six of the rabbits. Popliteal lymph nodes from both ipsilateral and contralateral sides were removed after death and examined at varying intervals after both primary and secondary immunization.

**Group I:** Preauricular lymph node stimulation: 24 rabbits were immunized by intradermal injection of Brucella antigen in two sites on the mid-dorsal aspect of the right ear. Preauricular lymph nodes were removed from rabbits killed at intervals following primary and secondary antigenic stimulation.

12 rabbits were immunized by intradermal injection of \(1 \times 10^{10}\) Brucella cells (0.05 ml) mixed with 0.05 ml of complete Freund’s adjuvant. 2 wk later, eight of these rabbits were given the same amount of Brucella antigen in saline without adjuvant, injected intradermally into the same ear.

12 rabbits were immunized with \(1 \times 10^{10}\) Brucella cells in saline intradermally in the right ear as above, and eight of these rabbits received a second, similar intradermal injection of Brucella antigen 2 wk later.

In each subgroup rabbits were killed on days 5 and 7 after primary stimulation (four animals) and on days 5, 10, 15, and 20 after secondary stimulation (eight animals).

**Group II:** Rabbits immunized with bovine serum albumin: 24 rabbits were immunized with BSA (Armour Pharmaceutical Company, Kankakee, Ill.) diluted in normal saline. Each rabbit was given 2 mg BSA in 0.1 ml of saline in divided doses intradermally in the ear as described above. 25 days later 12 of the animals were given 1 mg of BSA in 0.1 ml of saline intradermally in the same ear. These rabbits were killed on days 3, 5, 7, 10, 15, and 20 after either primary or secondary immunization.

**Group III:** Control nonimmunized rabbits: Nine rabbits, housed under similar conditions, were not immunized and served as controls. These animals were killed, and the popliteal lymph nodes from four rabbits and preauricular lymph nodes from five rabbits were removed.

Lymph nodes were prepared for study by routine histological methods and immunofluorescence.

**Immunofluorescence.**—Human and rabbit lymph nodes were quick-frozen in isopentane, cooled in liquid nitrogen, and sectioned in a Lipshaw cryostat at \(-30^\circ C\), using a method standard in our laboratory (22, 50). Serial frozen sections were fixed in acetone and stained with fluorescein-labeled antisera at room temperature by the method of Mellors (48). Specificity of the staining reactions was determined by absorption of the fluorescein-labeled antisera with its appropriate antigen.

**Elution Studies of Tissue Sections.**—Elution of human and rabbit lymph node tissue sections was performed by incubation at room temperature for 2-2.5 hr, using 0.05 m citrate-buffered saline, pH 3.3. Control sections were incubated for the same period of time in phosphate-buffered saline, pH 7.35, along with serial sections stained without incubation.

**Human Plasma Protein Antigens.**—Human plasma protein antigens, IgG, \(\beta_1 C\)-globulin, fibrinogen, and albumin were prepared by methods previously described by Michael et al. from our laboratory (50). In addition, human \(\beta_1 C\)-globulin was absorbed on zymosan particles using the method of Mardiney and Müller-Eberhard (46). Human IgM isolated by the method of Chaplin et al. (11) was generously donated by Dr. Richard Hong, Department of Pediatrics, University of Minnesota.

**Animal Plasma Protein Antigens.**—Rabbit IgG was isolated by column chromatography on DEAE-cellulose, and the Fc fragment used for immunization in goats was prepared by papain digestion (64) with the minor modifications of Ishizaka et al. (41). Rabbit IgM was isolated from sera harvested from rabbits stimulated with endotoxin by the method of Small and Lamm (66) and purified by the method of Chaplin et al. (11). Rabbit \(\beta_1 C\)-globulin was absorbed on zymosan particles using the method of Mardiney and Müller-Eberhard (46).
Goat IgG was prepared by chromatography on DEAE-Sephadex using 0.01 M phosphate buffer, pH 6.5.

Rabbit α2M was isolated by the following procedure. 350 ml of normal rabbit serum was centrifuged at 63,000 g for 20 hr in a Spinco ultracentrifuge. The protein pellet obtained was combined and suspended in saline and centrifuged at 40,000 g for 18 hr, and the resulting protein pellet was resuspended in a few drops of sodium barbital buffer (ionic strength 0.05, pH 6.8) and subjected to starch block electrophoresis at 18 hr at 4°C. Protein fractions from the eluted sections of the starch block which contained anodal migrating proteins and were shown by gel diffusion to be free of rabbit IgM were pooled and applied to a Sephadex G-200 column, using 0.1 M sodium acetate buffer, pH 4.0, containing 0.2 M sodium chloride. The first peak, containing heavy molecular weight material, shown to contain pure rabbit α2M as determined by acrylamide gel electrophoresis and double diffusion analysis, was used for immunization. Rabbit fibrinogen was isolated using the method of Blomback and Blombäck (5), and was generously provided by Dr. Roger Herdman, Department of Pediatrics, University of Minnesota.

Preparation of Antisera for Immunofluorescence Studies.—Antisera against human IgG, fibrin, β1G, albumin, and goat IgG were prepared in rabbits as previously described by Michael et al. (50). Antiserum against human IgM was prepared in goats and generously provided by Dr. Richard Hong.

Antisera against rabbit α2M, rabbit β1G-globulin, and rabbit fibrin were prepared in guinea pigs, and antisera against rabbit Fe fragment and rabbit IgM were prepared in goats. Antibody against BSA was prepared in rabbits (22, 50). All antisera were tested against normal human sera and normal rabbit sera and shown to be monospecific with the exception of the anti-fibrin antisera, which showed, besides the fibrin line, two or three additional lines to other serum proteins (these antisera were absorbed subsequently as described below). Immuno-electrophoretic analysis, kindly performed by Dr. Clark West, demonstrated that the antiserum prepared by immunisation with purified β1G contained antibody activity against both the A and B antigens of β1G-globulin, whereas the antiserum obtained by immunization with β1G absorbed on zymosan particles contained only the A antigen; neither of these antisera contained any detectable antibody activity to the α2M antigen (79). All antisera were precipitated with ammonium sulfate as previously described, and the IgG fraction was obtained by chromatography on DEAE-cellulose (67). This fraction was tagged with fluorescein isothiocyanate, using between 0.015 and 0.018 mg of fluorescein isothiocyanate/mg of protein. The mixture was chromatographed again on DEAE-cellulose and eluted in a stepwise fashion, using increasing concentrations of sodium chloride, by the method of Cebra and Goldstein (10, 83). Protein peaks with fluorescein/protein ratios of 5.0-9.0 X 10^-5, found to give low background and a high degree of specific staining, were used in these studies. The fluorescein-labeled proteins were adjusted to a concentration varying between 0.5 and 2.0 mg/ml and used in direct staining procedures.

For the detection of rabbit IgG only, indirect staining was employed, using first goat anti-rabbit Fe, followed by application of a second layer of fluorescein-labeled rabbit anti-goat IgG antiserum (48).

Goat anti-human IgM and goat anti-rabbit IgM labeled antisera were absorbed prior to staining, using 1 mg of purified human IgM and 1 mg of purified rabbit IgG, respectively, per milliliter of fluorescein-labeled antiserum. Similarly, the rabbit anti-human fibrin antiserum and the guinea pig anti-rabbit fibrin antiserum were absorbed with 1 mg of lyophilized normal human serum and normal rabbit serum, respectively.

In addition to demonstration of the staining specificity of the rabbit anti-human β1G antiserum by absorption with purified β1G antigen (kindly provided by Dr. H. J. Müller-Eberhard), the antiserum was absorbed with human β1G bound on BSA-anti BSA complexes,
which resulted in complete inhibition of staining. (Similar absorption with BSA—anti BSA complexes which had been incubated with fresh human serum in the presence of EDTA, as well as absorption with BSA—anti BSA complexes without human β1C, did not inhibit positive staining.)

Fixation of Heterologous Human Complement.—Sections of the same rabbit lymph nodes with germinal centers positive for β1C, BSA, and immunoglobulins were incubated for 30 min at 37°C with fresh human serum diluted 1:5 in phosphate-buffered saline. Serial control sections of the same node were incubated with the same human serum inactivated at 56°C, or with saline alone. The three incubated preparations were then stained simultaneously with fluorescent labeled antisera to human β1C, IgM, and IgG.

Routine Histology.—Serial cryostat sections were stained with hematoxylin and eosin. Part of each lymph node was also fixed in phosphate-buffered formalin, embedded in paraffin, and stained with hematoxylin and eosin.

RESULTS

Human Lymph Nodes.—β1C, IgM, and IgG in germinal centers were found in 21 of the 28 human lymph nodes studied. When examined in serial sections, the distribution of the β1C component of complement was identical with that observed for IgM and IgG (Figs. 3 and 4).

Well-developed germinal centers, strongly fluorescent when stained with anti-β1C, usually showed a strong fluorescence with both anti-IgG and anti-IgM. When β1C staining was weak, with a delicate reticular pattern, staining with anti-IgM was often more prominent than was staining for IgG. Usually these lymph nodes contained larger numbers of plasma cells, especially immature plasma cells producing IgM. Fibrin was present in lymphoid follicles of very congested lymph nodes only, and its distribution was completely different from that of IgG, IgM, and β1C (Fig. 5). Albumin was never observed in the lymphoid follicles investigated. Treatment of unfixed sections with citrate buffer at low pH resulted in marked diminution of IgG and IgM, and only some reduction of β1C.

14 mesenteric lymph nodes, seven mediastinal lymph nodes, and seven axillary and inguinal nodes were examined; the diagnoses of the patients from whom these nodes were obtained are shown in Table I. In 12 of the 14 mesenteric lymph nodes, β1C, IgG, and IgM were present in germinal centers. The remaining two lymph nodes examined, which lacked β1C, IgG, and IgM, were obtained from a 1 day old infant and a 27 year old agammaglobulinemic patient with a normal serum level of complement. Six of the seven inguinal and axillary lymph nodes contained germinal centers positive for β1C, IgG, and IgM. Three were obtained from patients with fatal granulomatous disease (32), and three had been stimulated with antigens to study antibody responses for diagnostic purposes. The single negative lymph node was obtained from the inguinal region of a healthy 9 year old girl who had received no stimulation. The node was obtained as a source of lymphoid cells to be given along with a bone marrow transplant to an hypogammaglobulinemic sibling. Three of seven
mediastinal lymph nodes were found to have germinal centers positive for \( \beta_{1C} \), IgG, and IgM. In each of these patients inflammatory changes were demonstrated in the lungs at autopsy.

### TABLE I

**Localization of \( \beta_{1C} \) IgG, and IgM in Germinal Centers of Human Lymph Nodes**

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age</th>
<th>Diagnosis</th>
<th>Lymph node</th>
<th>Presence of ( \beta_{1C} ), IgG, IgM</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. S. B.</td>
<td>1 day</td>
<td>Respiratory distress syndrome</td>
<td>Mesenteric</td>
<td>-</td>
</tr>
<tr>
<td>2. E. F.</td>
<td>8 months</td>
<td>Hypogammaglobulinemia (antigen stimulation)</td>
<td>Axillary</td>
<td>+</td>
</tr>
<tr>
<td>3. D. H.</td>
<td>1 yr</td>
<td>Immunological deficiency disease (antigen stimulation)</td>
<td>Axillary</td>
<td>+</td>
</tr>
<tr>
<td>4. S. O.</td>
<td>1½ yr</td>
<td>Congenital heart disease</td>
<td>Mesenteric</td>
<td>+</td>
</tr>
<tr>
<td>5. J. R.</td>
<td>2 yr</td>
<td>Hydrocephalus</td>
<td>Mesenteric</td>
<td>+</td>
</tr>
<tr>
<td>6. G. H.</td>
<td>5 yr</td>
<td>Fatal granulomatous disease</td>
<td>Inguinal</td>
<td>+</td>
</tr>
<tr>
<td>7. M. W.</td>
<td>5 yr</td>
<td>Aplastic anemia</td>
<td>Mesenteric</td>
<td>+</td>
</tr>
<tr>
<td>8. G. J.</td>
<td>8 yr</td>
<td>Mental deficiency</td>
<td>Mesenteric</td>
<td>+</td>
</tr>
<tr>
<td>9. J. R.</td>
<td>9 yr</td>
<td>Healthy (cell donor)</td>
<td>Inguinal</td>
<td>-</td>
</tr>
<tr>
<td>10. T. H.</td>
<td>11 yr</td>
<td>Fatal granulomatous disease</td>
<td>Inguinal</td>
<td>+</td>
</tr>
<tr>
<td>11. G. R.</td>
<td>12 yr</td>
<td>Brain tumor</td>
<td>Mesenteric</td>
<td>+</td>
</tr>
<tr>
<td>12. B. B.</td>
<td>14 yr</td>
<td>Diabetes mellitus</td>
<td>Mesenteric</td>
<td>+</td>
</tr>
<tr>
<td>13. J. K.</td>
<td>27 yr</td>
<td>Bruton-type hypogammaglobulinemia</td>
<td>Mesenteric</td>
<td>-</td>
</tr>
<tr>
<td>14. J. McD.</td>
<td>35 yr</td>
<td>Fatal granulomatous disease (?)</td>
<td>Inguinal</td>
<td>+</td>
</tr>
<tr>
<td>15. P. A.</td>
<td>40 yr</td>
<td>Aplastic anemia</td>
<td>Mesenteric</td>
<td>+</td>
</tr>
<tr>
<td>16. A. W.</td>
<td>42 yr</td>
<td>Liver cirrhosis</td>
<td>Mediastinal</td>
<td>-</td>
</tr>
<tr>
<td>17. M. N.</td>
<td>43 yr</td>
<td>Diabetes mellitus, pneumonitis</td>
<td>Mediastinal</td>
<td>+</td>
</tr>
<tr>
<td>18. H. H.</td>
<td>52 yr</td>
<td>Hypogammaglobulinemia (antigen stimulation)</td>
<td>Inguinal</td>
<td>+</td>
</tr>
<tr>
<td>19. B. H.</td>
<td>53 yr</td>
<td>Cardiac infarct</td>
<td>Mesenteric</td>
<td>+</td>
</tr>
<tr>
<td>20. G. H.</td>
<td>57 yr</td>
<td>Peritonitis</td>
<td>Mesenteric</td>
<td>+</td>
</tr>
<tr>
<td>21. G. S.</td>
<td>58 yr</td>
<td>Perforated gastric ulcer</td>
<td>Mesenteric</td>
<td>+</td>
</tr>
<tr>
<td>22. L. C.</td>
<td>59 yr</td>
<td>Cardiac surgery</td>
<td>Mediastinal</td>
<td>-</td>
</tr>
<tr>
<td>23. L. R.</td>
<td>61 yr</td>
<td>Rectal carcinoma, pneumonitis</td>
<td>Mediastinal</td>
<td>+</td>
</tr>
<tr>
<td>24. D. W.</td>
<td>65 yr</td>
<td>Cardiac failure, pneumonitis</td>
<td>Mediastinal</td>
<td>+</td>
</tr>
<tr>
<td>25. F. A.</td>
<td>70 yr</td>
<td>Parkinson's disease</td>
<td>Mesenteric</td>
<td>+</td>
</tr>
<tr>
<td>26. A. L.</td>
<td>72 yr</td>
<td>Pituitary adenoma</td>
<td>Mediastinal</td>
<td>-</td>
</tr>
</tbody>
</table>

**Lymph Nodes in Experimentally Stimulated Rabbits.**

**Group IA: Popliteal lymph node stimulation with Brucella.** Results of stimulation of lymph nodes with *Brucella* by the foot pad route were inconclusive, since early prominent formation of germinal centers in popliteal lymph nodes on the unstimulated side and well-developed germinal centers in unstimulated control
animals were observed. This finding suggested that very frequently spontaneous stimulation of popliteal lymph nodes had occurred, perhaps because of infection after superficial trauma. Both ipsilateral and contralateral nodes seemed to be stimulated, and even nodes of control animals often contained numerous germinal centers. To relate the germinal center response more certainly to defined antigenic stimulation, we turned to analysis of the response of preauricular nodes after intradermal injection of antigen into the ear.

**Group IB: Preauricular lymph node stimulation with Brucella:** On day 5 after primary stimulation, it was possible to localize $\beta_{\text{IC}}$, IgM, and IgG within the germinal centers of some follicles. The distribution of these proteins was always similar, most frequently in the form of delicate lacy structures, or sometimes as more granular accumulations. Lymph nodes stimulated with *Brucella* and Freund's adjuvant always contained considerable amounts of red cells in distended vessels, and featured more plasma cells than did lymph nodes stimulated with *Brucella* alone. On the seventh day after primary stimulation the picture was basically the same, with more numerous and better formed germinal centers regularly containing $\beta_{\text{IC}}$, IgM, and IgG.

After secondary stimulation, well-formed germinal centers were found on day 5 in both groups of rabbits (stimulated with *Brucella* and Freund's adjuvant and with *Brucella* alone). Approximately 30% of germinal centers contained $\beta_{\text{IC}}$, IgM, and IgG in a similar distribution. In contrast to the findings after primary stimulation, no differences in the number of plasma cells in the lymph nodes from these two groups of animals was apparent. The structure of lymph nodes stimulated with *Brucella* and Freund's adjuvant was not so well preserved as that of nodes stimulated with *Brucella* in saline, because of accumulations of many lipid-laden macrophages. The number of germinal centers positive for $\beta_{\text{IC}}$, IgG, and IgM increased on day 10 to approximately 60%, and persisted at this level when examined on days 15 and 20. Plasma cells were numerous up to 15 days after secondary stimulation. Changes in preauricular lymph nodes on both stimulated and unstimulated sides were similar, a finding we attributed to the high doses of antigen used.

**Group II: Rabbits immunized with BSA; Germinal centers.** The appearance of BSA, $\beta_{\text{IC}}$, and immunoglobulins in germinal centers (identified in serial sections stained with hematoxylin and eosin) is shown in Figs. 1 and 2. The numbers express the mean percentage of positive germinal centers found in two rabbits killed on the same day. Since the percentage of germinal centers positive for IgG and IgM varied no more than 15% (usually less than 5%), we have combined the data for IgG and IgM to simplify graphic representation.

After primary stimulation, the localization of $\beta_{\text{IC}}$ and immunoglobulins reached a peak between the fifth and 10th days and decreased gradually over the ensuing 10 days. Maximal localization of BSA occurred in the germinal centers on the 10th day after antigenic stimulation and decreased in parallel
with the other components (Fig. 1). A peak percentage of germinal centers positive for $\beta_{IC}$, BSA, and immunoglobulins was reached on day 7 after secondary stimulation. Subsequently the number of germinal centers positive for BSA decreased significantly, in contrast to the percentage of germinal centers positive for $\beta_{IC}$ and immunoglobulins, which persisted up to day 20 (Fig. 2).

![Diagram](attachment:image.png)

**Fig. 1. Localization of $\beta_{IC}$, immunoglobulins, and antigen in rabbit lymph nodes after primary stimulation with BSA.**

As in group I, there was a striking and consistent correlation between distribution and appearance of specific staining for BSA, $\beta_{IC}$, IgG, and IgM in germinal centers (Fig. 6–8). These deposits appeared to be located on the surface of reticulum cells and on their dendritic processes, giving a characteristic reticular or granular pattern. Similar staining with fluorescein-labeled BSA was observed, which suggested that at least some of the immunoglobulins within germinal centers possessed specific anti-BSA activity. As in the material from human patients, the localization of fibrin was always different from that of $\beta_{IC}$, IgM, and IgG and was observed primarily in lymph nodes with vascular conges-
tion. $\alpha_{2M}$ was not found in germinal centers. Deposits of the immune reactants in germinal centers frequently showed a "crescentic" distribution occupying an area which was identified by light microscopy as a zone of lighter staining dendritic reticulum cells and relatively few lymphoid cells (Fig. 9).

**SECONDARY STIMULATION**

![Graph showing the localization of $\beta_{1C}$ immunoglobulins, and antigen in rabbit lymph nodes after secondary stimulation with BSA.]

**Fig. 2.** Localization of $\beta_{1C}$, immunoglobulins, and antigen in rabbit lymph nodes after secondary stimulation with BSA.

*Elution Studies and Fixation of Heterologous Complement.*—Treatment of unfixed sections with citrate buffer at low pH resulted in complete elution of immunoglobulins, $\beta_{1C}$, and BSA from rabbit lymph node germinal centers (Fig. 10). $\beta_{1C}$ granules within macrophages in the medullary part of the node were almost completely eluted as well. However, IgG and IgM within plasma cells were not eluted (Fig. 11). No elution was observed in sections incubated with saline.

After exposure of rabbit lymph node sections to fresh human serum, it was
possible to demonstrate specific fixation of heterologous complement in germinal centers. Human $\beta_{1c}$ was observed in the same distribution as rabbit $\beta_{1c}$ and immunoglobulins (Fig. 12). Control sections incubated with heat-inactivated human serum were negative, as were all sections stained for IgG and IgM.

In group II, preauricular lymph nodes on the unstimulated side often did not show formation of germinal centers. When germinal centers were present, although in very reduced numbers in the contralateral node, it was possible to demonstrate IgG, IgM, and $\beta_{1c}$ in the same area. In contrast to the ipsilateral node, minimal deposits of BSA were present in the germinal centers of the contralateral node.

**Plasma Cells.**—Plasma cells were most numerous on day 5 and were located mainly in medullary cords, while later, at days 15 and 20, they accumulated around secondary follicles, primarily adjacent to the medulla of the node.

**Lymphocytes.**—The lymphocytes in primary follicles, diffuse cortex, and medullary cords showed faint cytoplasmic fluorescence with anti-IgG and anti-IgM between days 3 and 7 after primary stimulation and on day 3 after secondary immunization (Fig. 13). (Similar, faintly fluorescent lymphocytes were observed in human lymph nodes containing early germinal centers and numerous immature plasma cells.) At later stages of lymph node stimulation, germinal centers positive for $\beta_{1c}$ and immunoglobulins were surrounded by an inner ring of “dark” or nonstaining lymphocytes, and an outer ring of fluorescent lymphocytes and plasma cells (Fig. 14).

**Eosinophils.**—Eosinophils were often noted in maximal numbers on day 3 after both primary and secondary stimulation, and were located mainly at the periphery of follicles and in medullary regions of lymph nodes. Between days 7 and 10 after stimulation, the number of eosinophils decreased.

**Macrophages.**—In some lymph nodes, mainly in the early period after stimulation but infrequently as late as the 20th day, macrophages lining medullary sinuses contained small, brightly fluorescent granules when stained for $\beta_{1c}$. Some of these macrophages contained other granules with orange-red autofluorescence. It was difficult to determine whether $\beta_{1c}$ was located in the cytoplasm or on the surface of such cells. In a single rabbit, on day 3 after primary stimulation, small granules of BSA were observed within macrophages in the medullary part of the lymph node. “Tingible body” macrophages were present in the germinal centers during all periods of observation and contained orange autofluorescent granules. Some of these also seemed to stain weakly for BSA and $\beta_{1c}$.

**DISCUSSION**

These observations establish, in agreement with the findings of others (7, 57, 61), that germinal centers of human lymph nodes regularly contain IgG and IgM. In addition, our work confirms the observations of Nowoslawski and
Brzosko\textsuperscript{1} that germinal centers contain $\beta_{I\text{C}}$. After antigenic stimulation with either \textit{Brucella} antigen or BSA, germinal centers in rabbit lymph nodes contained $\beta_{I\text{C}}$, IgG, IgM, BSA, and antibodies to BSA. All these components were shown to be present in the same distribution within germinal centers; we feel that this presents evidence of an antigen-antibody complex mechanism. Furthermore, the finding that additional exogenous $\beta_{I\text{C}}$ is bound in the same distribution within germinal centers of rabbit lymph nodes after treatment with fresh human serum establishes that immunological complexes actually fix heterologous complement components in vitro. Attempts to elute complexes from germinal centers of human lymph nodes revealed significant diminution of IgG and IgM, but only minimal reduction of $\beta_{I\text{C}}$. However, elution of rabbit lymph node sections resulted in complete elimination of IgG, IgM, $\beta_{I\text{C}}$, and BSA from germinal centers. In contrast, the IgG and IgM content of plasma cells was not altered by this treatment.

These findings permit the conclusion that IgG, IgM, and $\beta_{I\text{C}}$ are located on the surface of cells within germinal centers of rabbits after antigenic stimulation, and are present in the form of complexes. They also suggest that, at least for 20 days after either primary or secondary antigenic stimulation, IgG, IgM, and $\beta_{I\text{C}}$ are not synthesized by cells in the germinal centers but are deposited as antigen-antibody complexes with $\beta_{I\text{C}}$. Furthermore, elution experiments seem to indicate that the bulk of $\beta_{I\text{C}}$ can be eluted from macrophages in the medulla of the lymph node; this finding is somewhat incongruous with observations of others, who have presented evidence of complement component synthesis in lymph nodes (3, 23, 26, 39, 65, 68–72, 74).

It seems unusual that following deposition of biologically active antigen-antibody complexes with $\beta_{I\text{C}}$, little tissue injury occurs in germinal centers (13, 24, 40, 58, 60, 78). This observation cannot be explained on the basis of unfavorable antigen-antibody ratios, because we have shown $\beta_{I\text{C}}$ to be present at the same sites as the antigen-antibody complexes and have demonstrated fixation of exogenous $\beta_{I\text{C}}$ in vitro. Perhaps attachment of the complexes to the surface of dendritic macrophages partially inhibits chemotactic influence, which would otherwise be expected to operate when antigen-antibody and complement are deposited in tissues. Alternatively, it is possible that activation of the more terminal components of complement, such as C5 or the C5,6,7\textsuperscript{2} trimolecular complex (55), does not take place under the conditions prevailing in germinal centers.

Much evidence indicates that lymphocytes, perhaps of a special thymus-independent line of differentiation (31), proliferate in germinal centers (25), and it has been proposed that antigen is located on the surface of the dendritic

\textsuperscript{1} Nowoslawski, A., and W. J. Brzosko. Unpublished observations.

\textsuperscript{2} Complement nomenclature according to World Health Organization.
reticulum cells to stimulate lymphoid cells at these sites (20, 51, 52, 56). Our findings are consonant with this view. The known ability of complement to alter cell membranes suggests that the complexes could alter either the membranes of lymphocytes or dendritic macrophages to contribute to this postulated differentiative influence.

Evidence has also been presented that germinal centers are linked in some way to the enhanced antibody synthesis associated with immunological memory (27, 76, 77). Immunological memory, as usually defined, is known to be associated with synthesis of IgG molecules (27). Investigations from both the phylogenetic and the ontogenetic perspective link germinal centers to the synthesis and delivery of IgG (27–29, 63). For example, chickens bursectomized at 18–19 days in ovo can produce abundant IgM and possess plasma cells, but lack IgG and germinal centers (9, 18). Perhaps germinal centers function chiefly either as sites of further differentiation of the line of thymic independent immunoglobulin-producing cells or as sites capable of exercising an inductive influence in this direction on surrounding lymphocytes. In the latter case, antigen-antibody complexes, particularly in the presence of complement, could play an inducing role different from that exercised by antigen alone. This type of reaction within the germinal center could, for example, stimulate lymphoid cells to proliferate without maturing to immunoglobulin secretion. After some proliferation of an apparently wasteful nature at this site, “antigen-insensitive” cells could effect a derepression and then become “antigen-sensitive” cells capable of IgG synthesis and secretion.

The faint staining of lymphocytes for IgG and IgM in the mantle zones about germinal centers and the development of plasma cells in these regions are of interest. Such lymphocytes faintly staining for immunoglobulins appear prior to the development of germinal centers. Morphological evidence for transformation of lymphocytes into plasma cells between days 1 and 4 following antigenic stimulation has been reported in rabbit lymph nodes (43) and spleen (44), with subsequent development of germinal centers. Similar findings, using electron microscopic techniques, have demonstrated that in rabbit lymph nodes lymphocyte transformation to plasma cells begins in the diffuse cortex before development of the germinal centers (53). Recently Bosman et al. showed by radioautographic techniques and electron microscopy that after stimulation of rats with BSA lymphocytes constituted the majority of cells producing specific antibodies in draining lymph nodes (6).

In this study, the role of germinal centers in immune responses and the body economy still remains enigmatic. Understanding of this role must take into account the facts that germinal centers contain antigen-antibody complexes with complement, and that for a prolonged period after both primary and secondary antigenic stimulation such complexes account for the bulk of the immunoglobulins and probably the bulk of antibodies at these sites.
28 human and 60 experimentally stimulated rabbit lymph nodes were studied by means of light microscopy and immunofluorescence. 21 of the 28 human lymph nodes showed well-developed germinal centers. IgM, IgG, and the $\beta_{1C}$ component of complement were found in the same distribution within germinal centers when examined in serial cryostat sections. 36 rabbits were stimulated with Brucella antigen, and 24 rabbits with BSA. A strikingly consistent correlation between distribution and appearance of specific staining for rabbit $\beta_{1C}$, IgM, and IgG was observed; when lymph nodes were stimulated with BSA, antigen and specific antibody were present. Treatment of unfixed sections with citrate-buffered saline at low pH resulted in complete elution of immunoglobulins, $\beta_{1C}$, and BSA from rabbit germinal centers, and in marked diminution of IgG and IgM in human germinal centers, while at the same time plasma cells remained strongly fluorescent. Specific selective fixation of heterologous (human) complement in rabbit germinal centers positive for $\beta_{1C}$, IgG, IgM, and BSA was also obtained.

These data present strong evidence for the existence within germinal centers of antigen-antibody complexes which fix at least the $\beta_{1C}$ component of complement in vivo. The possibility of complete elution of immunoglobulins from rabbit germinal centers can be taken as evidence that, at least for 20 days after primary and secondary stimulation, a major component of the immunoglobulins present in germinal centers is not produced locally but accumulates at the surface of cells.

We would like to acknowledge the valuable help and advice provided in this work by Dr. A. Michael, Dr. W. Brzosko, Dr. A. Nowoslawski, Dr. R. Pickering, Dr. R. Hong, and Dr. H. Gewurz. We are indebted to Dr. Hans J. Müller-Eberhard, who provided purified human $\beta_{1C}$ for this study and reviewed the manuscript. The technical assistance of Mrs. Lore Lang, Miss Agnes Opstad, and Miss Mary Ann Morris and the secretarial assistance of Mrs. Betty Duquesnoy are greatly appreciated.

BIBLIOGRAPHY

β_{BC} FIXATION IN LYMPH NODE GERMINAL CENTERS


34. Hanna, M. G., Jr. 1964. An autoradiographic study of the germinal center in...


1384 βc FIXATION IN LYMPH NODE GERMINAL CENTERS


Fig. 3. Localization of human $\beta_{1C}$ in a germinal center of a mesenteric lymph node (case 8). $\times$ 200.

Fig. 4. Localization of human IgM in a germinal center of a mesenteric lymph node (case 8), serial section of Fig. 1. $\times$ 200.

Fig. 5. Staining for human fibrin in a mesenteric lymph node. The localization of the fluorescence is around the germinal center, and thus different from the localization of IgM and $\beta_{1C}$ (case 8). $\times$ 200.
FIG. 6. Distribution of BSA in a germinal center of a rabbit preauricular lymph node 7 days after secondary stimulation. BSA is found predominantly in between the cells (arrow). Bright, nonspecifically fluorescent eosinophils can be seen at the periphery of the follicle. × 200.

FIG. 7. Distribution of IgM in a germinal center of a rabbit preauricular lymph node 7 days after secondary stimulation with BSA. × 200.

FIG. 8. Distribution of $\beta_{1c}$ in a germinal center of a rabbit preauricular lymph node 15 days after secondary stimulation with BSA. Bright, nonspecifically fluorescent eosinophils are interspersed with cells staining specifically for $\beta_{1c}$. × 200.
FIG. 9. Crescentic distribution of IgM in a germinal center of a rabbit preauricular lymph node 10 days after primary stimulation with BSA. This crescentic distribution is noted in some instances in germinal centers early after primary stimulation. × 200.

Fig. 10. Elution of IgG from a germinal center following incubation in citrate-buffered saline, pH 3.3. Very little specific fluorescence remains in the germinal center after this procedure. In the periphery of the follicle and in medullary cords, plasma cells remain strongly fluorescent (15 days after secondary stimulation with BSA). × 200.

Fig. 11. Serial section of the same lymph node as in Fig. 10, incubated in phosphate-buffered saline, pH 7.35, and stained with anti-rabbit IgG. IgG is not eluted from the germinal center. Intensity of fluorescence of plasma cells is almost the same as in Fig. 10. × 200.
**Fig. 12.** Fixation and distribution of heterologous (human) 
\( \beta_{1C} \) in a germinal center 
of a rabbit preauricular lymph node 15 days after secondary stimulation with BSA 
(compare with Fig. 8). \( \times 200 \).

**Fig. 13.** Faint fluorescence of lymphocytes in a primary follicle (arrow), 
stained with anti-rabbit IgG. The strong bright fluorescence of plasma cells in medullary 
cords is evident (5 days after primary stimulation with \textit{Brucella} alone). \( \times 200 \).

**Fig. 14.** Lymphoid follicles from lymph node 15 days after secondary stimulation 
with \textit{Brucella}, stained for IgG. In the center of the two follicles the germinal centers 
are brightly fluorescent. The zone surrounding the germinal center consists of two 
parts: a dark inner zone, and an outer zone containing fluorescent plasma cells and 
lymphocytes. \( \times 200 \).