

Human immune response to pneumococcal polysaccharides: Complement-mediated localization preferentially on CD21-positive splenic marginal zone B cells and follicular dendritic cells

**María-José Peset Llopis, MSc, Geert Harms, BSc,
Machiel J. Hardonk, PhD, and Wim Timens, MD, PhD**
Groningen, The Netherlands

A functionally intact spleen with a marginal zone, containing B cells with high density of surface C3d-receptors (CD21), is essential for the ability to induce a primary immune response to thymus-independent type 2 (TI-2) antigens. Main representatives of natural TI-2 antigens are capsular pneumococcal polysaccharides (PPSs). In this study the localization of different types of PPS antigen is determined in human spleen tissue. Our findings indicate that a main type of TI-2 antigen, PPS, localizes preferentially in the marginal zone. PPSs show co-localization with C3, presumably C3d, at the surface of strongly CD21+ B cells equipped for rapid activation. This enables a rapid primary humoral response. The other main PPS localization at follicular dendritic cells in germinal centers, relevant for isotype switching of anti-PPS antibodies, does not seem to be dependent on the presence of specific immunoglobulin. This may explain the finding of specific IgG in an early stage after antigenic challenge. It seems likely that complement C3 fragments (likely C3d), bound to PPSs, enable PPS localization at B-cell and follicular dendritic cell surfaces by binding to CD21, the C3d receptor. (J ALLERGY CLIN IMMUNOL 1996;97:1015-24.)

Key words: CD21, CR2, follicular dendritic cells, marginal zone, pneumococcal polysaccharides, spleen, TI-2 response

The spleen plays an essential role in the defense against encapsulated bacteria (e.g., *Streptococcus pneumoniae*, *Hemophilus influenzae*, and *Neisseria meningitidis*).^{1,2} For the induction of a primary immune response against these microorganisms, a normal spleen function is necessary³; the presence of an unimpaired marginal zone is crucial.¹⁻⁶ The immune response is mainly directed against the polysaccharides located in the bacterial capsule, classified as thymus-independent type 2 (TI-2) antigens.^{1-4, 6, 7} For a humoral immune response

Abbreviations used

CR:	Complement receptor
FDCs:	Follicular dendritic cells
GC:	Germinal center
IHS:	Inactivated human serum
mAb:	Monoclonal antibody
NHS:	Normal human serum (fresh)
PALS:	Periarteriolar lymphocyte sheath
PPS:	Pneumococcal polysaccharide
TI-2:	Thymus-independent antigen type 2

From the Department of Pathology, University of Groningen. Supported by the Groningen Foundation for Pediatric Oncology.

Received for publication Feb. 16, 1995; revised June 6, 1995; accepted for publication June 14, 1995.

Reprint requests: Wim Timens, MD, PhD, Department of Pathology, University of Groningen, Oostersingel 63, NL-9713 EZ Groningen, The Netherlands.

Copyright © 1996 by Mosby-Year Book, Inc.

0091-6749/96 \$5.00 + 0 1/1/67054

against these antigens, the actual presence of T cells is not necessary, but T-cell factors are required.¹ Contrary to the primary immune response, a secondary immune response can be evoked outside the spleen.^{2,8}

Both patients with an anatomic asplenia and patients with a functional asplenia have a higher risk of a fulminant infection with encapsulated

bacteria.^{2,6} Children younger than 2 years of age also have a higher risk because of immaturity of spleen function.^{1,4,5,7} During development, B cells and T cells in the spleen gain more mature features.^{1,5} The turning point in the defense against TI-2 antigens at the age of 2 years takes place just when the CD21 surface molecule (C3d/Epstein-Barr virus receptor)^{9,10} appears in relatively high density on the B cells in the marginal zone. Before the age of 2 years, low or absent CD21 points to the existence of a marginal zone "early B cell" that has not yet matured.^{1,5,11-13}

The relation of the ability to induce a primary TI-2 antigen response and the presence of B cells with high CD21 density is of even more interest because the complement system, particularly complement fragment C3d, is reported to play an important role in the activation of B cells in the spleen during a primary immune response against TI-2 antigens.^{1,2,5,7,11} Moreover, the C3d fragment is able to bind to the pneumococcal polysaccharide (PPS); in this way, these complexes can be recognized by the complement receptor type 2 (CR2).¹¹

In contrast to most other studies,^{2,4,14} in this study we have used purified PPS as a natural type of TI-2 antigen. Eighty-three PPS types are defined serologically, of which 23 are used in the current vaccine, Pneumovax (Merck, Sharp, and Dohme, West Point, Pa.).⁷ To study the PPS localization patterns and cellular molecules that may play a role in localization, we incubated different types of PPS on human spleen sections in the presence of complement-containing serum or complement-inactivated serum. Influences of systemic diseases on the localization of the PPS types in the human spleen were studied with the use of spleens from patients with systemic disease in comparison with spleens obtained from patients with trauma injuries.

METHODS

Human spleen, lymph node, and tonsil tissue

For PPS binding studies, frozen tissue blocks of human spleens that showed no histologic abnormalities in routine histopathologic evaluation were used. Spleens were obtained from patients after a trauma injury ($n = 4$; 1 woman, age 30 years; and 3 men, ages 17, 35, and 62 years) and from patients younger than 19 years of age who had undergone splenectomy because of staging for Hodgkin's disease ($n = 6$), idiopathic thrombocytopenic purpura ($n = 2$), or a hemoglobinopathy (Hb Wolga

($n = 2$). Six spleens were obtained from nonvaccinated patients, two spleens were from patients who were vaccinated before surgery was performed, and six were from patients for whom data regarding vaccination were unknown.

To evaluate the supposed role of CD21 in the localization of PPSs, we studied spleens of newborns ($n = 4$, 38 to 42 weeks old) and infants ($n = 5$, 6 to 14 months old), because CD21 expression is low or absent at this age.¹ For comparison, localization of PPSs was studied in reactive lymph nodes ($n = 3$) and tonsils ($n = 2$).

Sera

Blood was obtained by venipuncture from healthy male and female adult donors. Clotting was allowed at 0° C for 2 hours to reduce complement activation. The blood was centrifuged at 1000 g for 10 minutes at 4° C. Part of this normal human serum (NHS) was stored at -70° C in aliquots of 200 μ l until use. The rest of the serum was heated for 30 minutes at 56° C to inactivate complement; this inactivated human serum (IHS) was stored at -70° C until use.

Antigens

For in vitro localization studies, PPS types 1, 2, 3, 4, 6A, 6B, 9N, 9V, 10A, 12F, 14, 19A, and 19F (American Type Culture Collection, Rockville, Md.) were diluted in distilled water to a final concentration of 1 mg/ml.

In the evaluation of newborn and infant spleens, we used PPS types representative of different "patterns" observed in adult spleens: PPS2, PPS10A, PPS14.

Antibodies

Rabbit anti-human C3d immunoglobulins (anti-C3d), peroxidase-conjugated rabbit anti-mouse immunoglobulin antiserum, and peroxidase-conjugated swine anti-rabbit immunoglobulin antiserum were obtained from Dakopatts (Glostrup, Denmark). To detect the different types of PPS antigens, type- or group-specific rabbit anti-PPS immunoglobulins were used (State Serum Institute, Copenhagen, Denmark).

Anti-CD2 (Leu-5b) and anti-CD3 (Leu-4) monoclonal antibodies (mAbs) were used to detect all T cells. Anti-CD20 (B-ly1) and anti-CD22 (Leu-14) mAbs were used to detect B cells. Anti-CD21 (HB5) is a specific antibody for the C3d/EBV receptor (CR2). Anti-CD35 (CR1), which detects the C3b receptor, was obtained from DAKO (Glostrup, Denmark).^{9,10} B-ly1 was developed at our own laboratory and included in the Fourth Leucocyte Typing Conference¹⁵; all other antibodies were from Becton-Dickinson (San Jose, Calif.). An immunoperoxidase staining procedure was applied as follows. Sections, air-dried for 20 minutes and fixed in acetone (100%) for 10 minutes at 20° C, were incubated for 30 minutes with the different mAbs at optimal dilutions, as determined separately. Next, sections were incubated with peroxidase-conjugated rabbit anti-mouse

TABLE I. Localization of PPS and distribution of complement receptors CR1/CD35 and CR2/CD21 in adult human spleen

PPS types	GC*	Mantle zone	Marginal zone	Red pulp
PPS1, 2, 3, 6A, 6B, 9V, 9N, 12F, 14, 19A, 19F				
NHS	++	+	++	—
IHS	—	—	—	—
PPS4				
NHS	+++	—/±	—/±	—
IHS	±	—	—	—
PPS10A				
NHS	+	—	—	—
IHS	—	—	—	—
CD21	+++	+	++	—
CD35	+++	++	++	+ / + + gran

*This is in fact FDC staining; GC B cells seem negative.

immunoglobulin antiserum diluted 1:20 in phosphate-buffered saline with 5% human AB serum for 15 minutes. Each incubation step was followed by rinsing the sections with phosphate-buffered saline for 5 minutes, with three changes. The peroxidase activity was visualized with 3-amino-9-ethylcarbazole and H₂O₂. Finally, sections were washed in distilled water and counterstained in Mayer's hematoxylin, rinsed in running tap water, and covered with Kaiser's glycerin-gelatin and a coverslip.

Detection of PPS binding

Cryostat sections (4 µm) of the human spleens were picked up on glass slides and kept at –20° C until use. Different types of PPS were preincubated for 1 hour at 37° C in a dilution of 1:4 (0.25 mg/ml) in NHS or IHS, respectively. An immunoperoxidase procedure was performed; it was similar to that described above, except that peroxidase-conjugated swine anti-rabbit immunoglobulin antiserum was used instead of peroxidase-conjugated rabbit anti-mouse immunoglobulin antiserum for the detection of the rabbit antisera. Sections, air-dried for 20 minutes and fixed in acetone (100%) for 10 minutes at 20° C, were incubated for 30 minutes at 37° C with the different types of PPS, preincubated in NHS or IHS, respectively. Sections were then incubated for 30 minutes with the different type- or group-specific rabbit antisera (State Serum Institute): anti-PPS 1, 2, 3, 4, 6, 9, 10, 12, 14, and 19, respectively. These sera were used at optimal dilutions, as determined separately, with 5% human AB serum (heat-inactivated). As controls for the immunohistologic staining procedure, sections were used in which the incubation with PPS or with PPS-specific antiserum was omitted or in which the type-specific anti-PPS serum was replaced, for this study, with nonrelevant rabbit antisera against factor VIII or CD3 (both from DAKO).

Complement fragment C3d

To further study the role of complement fragments in the binding of PPSs, we repeated the procedure used for the detection of PPS binding on human spleen sections, but instead of anti-PPS we used anti-C3d antiserum (DAKO, diluted 1:100). This antibody is known to react with epitopes expressed on C3, C3b, iC3b, and C3d.g.¹⁶ To evaluate localization of the recognized complement fragment without the presence of PPS, these sections were compared with sections in which the incubation with PPS antigens was omitted.

Double staining procedures

The double staining procedures for PPSs and relevant leukocyte antigens were performed with polyclonal fluorescein-conjugated goat anti-rabbit immunoglobulin antiserum (Southern Biotechnology Associates, Birmingham, Ala.), which detects rabbit anti-PPS immunoglobulin, and rhodamine-conjugated goat anti-mouse immunoglobulin antiserum (Southern Biotechnology Associates), which detects the mouse mAb against CD20, CD21, and CD35. Slides were evaluated under a fluorescence microscope with appropriate red and green filters.

RESULTS

PPS localization in adult spleen, lymph node, and tonsil tissue

Table I summarizes the results in spleen tissue. Staining results, depicted semiquantitatively, were consistent and reproducible. Staining patterns obtained with PPS types were compared with CD2/3-, CD20/22-, CD21-, and CD35-stained sections; the latter three were also compared in double staining procedures (Figs. 1 and 2). CD2/3 and CD20/22 were used to delineate T- and B-cell compart-

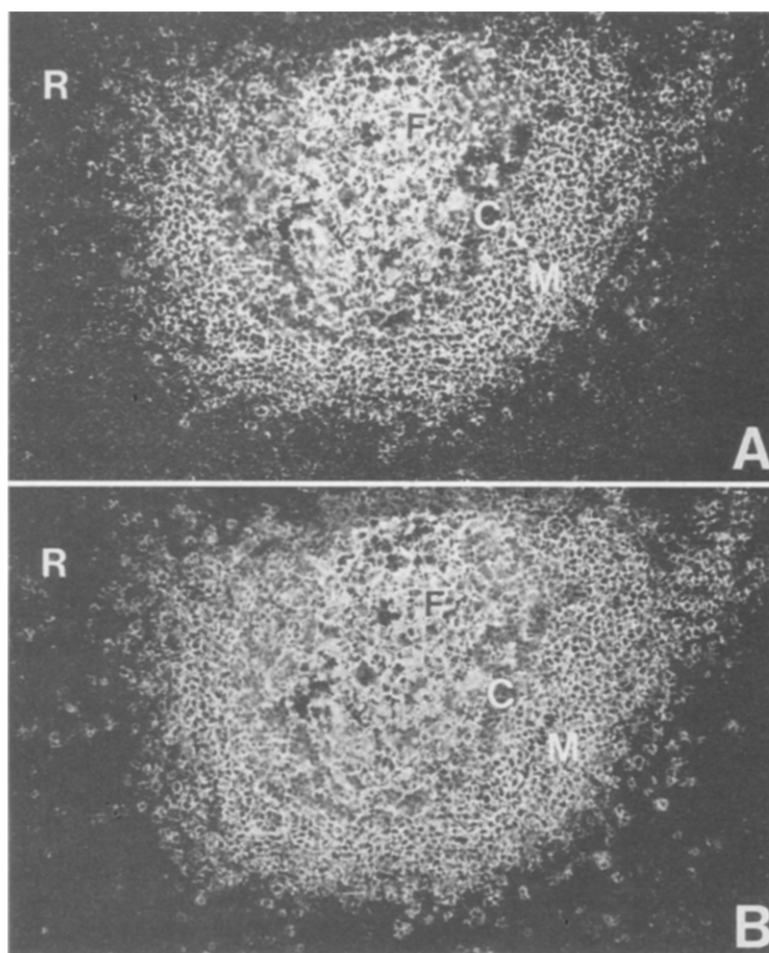


FIG. 1. Normal human spleen excised because of trauma; double immunofluorescence for PPS2 and CD21 (all $\times 140$). **A**, Localization of PPS type 2. Note strong FDC staining and staining of marginal zone B cells. Between the marginal zone and the GC, a small rim of weaker staining the mantle zone or corona B cells is seen. **B**, CD21 reactivity, similar to pattern observed in **A**. *M*, Marginal zone; *C*, lymphocyte corona; *F*, follicle center; *R*, red pulp.

ments. In all spleens, these show an expected pattern.¹⁷ CD21 and CD35 show staining of follicular dendritic cells (FDCs) and B cells from the mantle zone (CD21 stains rather weakly) and the marginal zone (Figs. 1 and 2). CD35 reacts, in addition, with granulocytes and more weakly with histiocytes/monocytes.

Sections that were stained with NHS incubated PPS revealed a common staining pattern for all types of PPS for all spleens obtained from nonvaccinated adult patients (Figs. 1, *A*, and 2, *A*). In spleens from the two vaccinated patients, patterns were similar but showed variation in staining intensity between different PPS subtypes. PPS binding was seen in germinal centers (GCs), mantle zones, and marginal zones. GCs showed a strongly stained meshwork pattern, identical to the CD21

and CD35 pattern as confirmed by double immunofluorescence. Double staining for IgD and PPS showed that this meshwork staining coincided with the IgD⁺ central area of B-cell follicles, as is characteristic for GCs. Primary follicles show IgD⁺ staining of all B cells. Mantle zones and marginal zones, the first staining more weakly than the latter, showed membrane staining of individual lymphocytes. In double staining procedures all cells with surface localization of PPS were labeled with CD20, CD21, and CD35. However, not all CD20⁺, CD21⁺, CD35⁺ B cells showed PPS localization. CD35⁺, CD20⁺, CD21⁺ cells in the red pulp, presumably granulocytes, never showed double labeling with PPS. The periarteriolar lymphocyte sheath (PALS) and the red pulp were negative. In lymph nodes and tonsils, mainly a

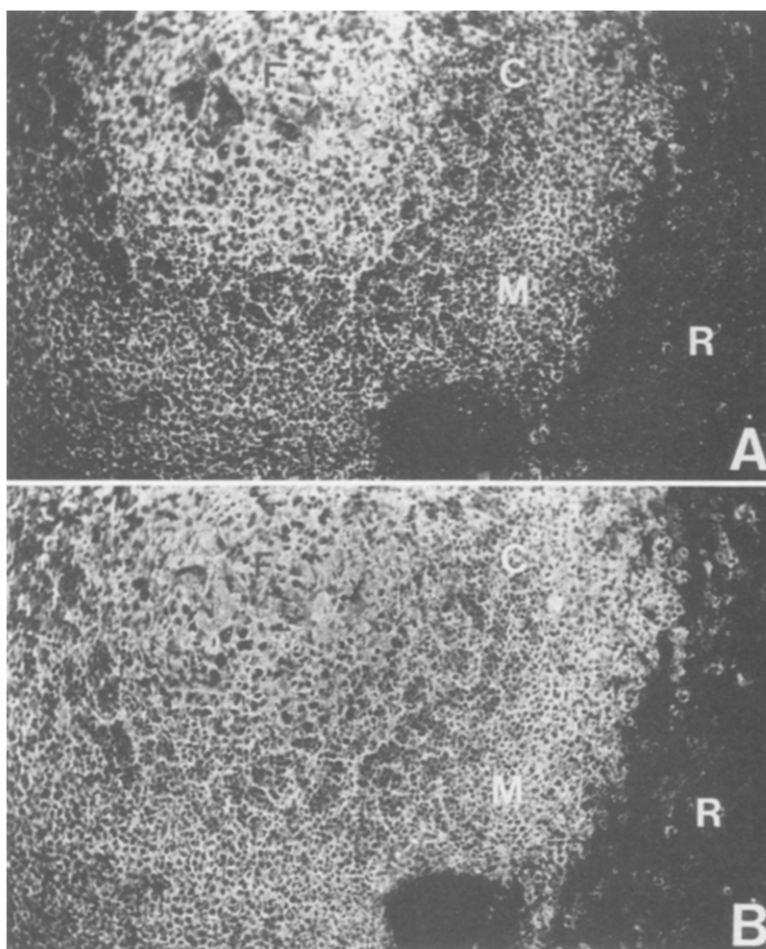


FIG. 2. Normal human spleen excised because of trauma; double immunofluorescence for PPS2 and CD35 (all $\times 140$). **A**, Localization of PPS type 2 (similar to Fig. 1, A). **B**, Staining for CD35 (CR1) with similarity to A, but with additional perifollicular cells, mostly neutrophils (arrows). M, Marginal zone; C, lymphocyte corona; F, follicle center; R, red pulp.

meshwork pattern of GCs was observed, with very low reactivity of mantle zone cells (Fig. 3).

A few exceptions to these common patterns were seen. In contrast to other PPS types, anti-PPS 10A and anti-PPS 4 showed only weak staining for all spleens; anti-PPS 10A sometimes showed negative staining. Anti-PPS 4 showed staining of the red pulp sinusoid lining cells, which were clearly delineated. In the staining procedure in which inactivated serum was used, sections of spleens from patients who were not vaccinated before surgery were completely negative. Spleens from the two vaccinated patients showed PPS types 2, 6, 9V, 19A, and 19F with positive GCs (Fig. 4). The mantle zone, the marginal zone, PALS, and red pulp were negative. Spleens from patients for whom data regarding vaccination were unknown

all showed results similar to those of the nonvaccinated patients.

No differences were found between spleens from trauma patients and those from patients with systemic diseases. Results of controls with omission of the PPS showed results similar to those obtained with PPS incubated with inactivated serum. Replacement of rabbit anti-PPS antiserum by other rabbit antisera resulted in negative staining of FDCs and the B-cell area, which showed positive staining with the specific antisera.

Newborn and infant spleens

In newborn spleens in general no marginal zone area is present.¹⁷ In infant spleens a marginal zone is present with low or absent expression of CD21¹ but with presence of CD35 on the marginal zone

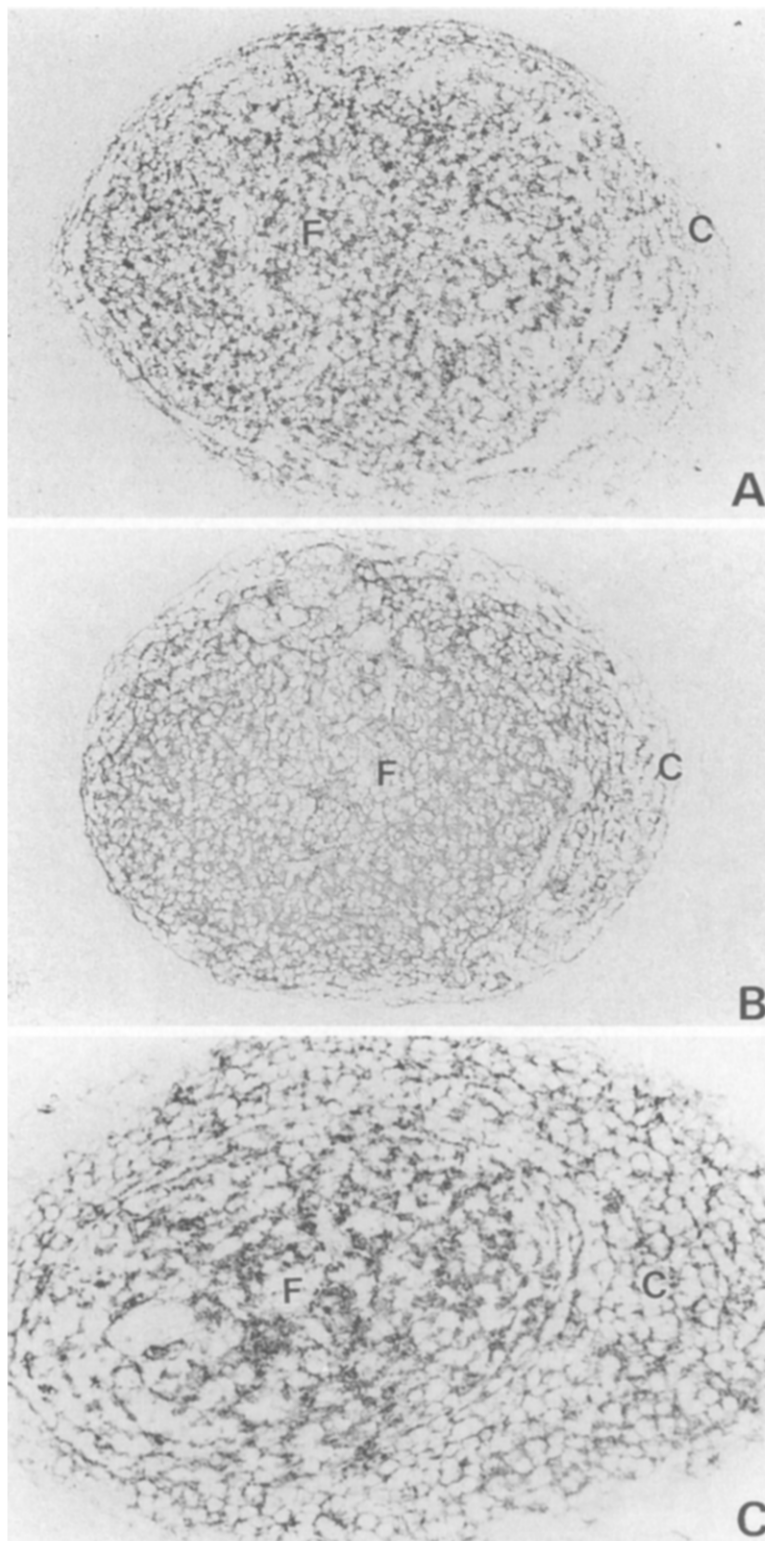


FIG. 3. Normal human lymph node. **A**, Localization of PPS type 14. Note strong FDC staining without staining of mantle zone/corona B cells ($\times 140$). **B**, CD21 reactivity, similar to pattern observed in **A** ($\times 140$). **C**, Staining for CD35 (CR1) with similarity to **A** and **B**, but with stronger staining of mantle zone/corona B cells ($\times 350$). **C**, Lymphocyte corona; **F**, follicle center.

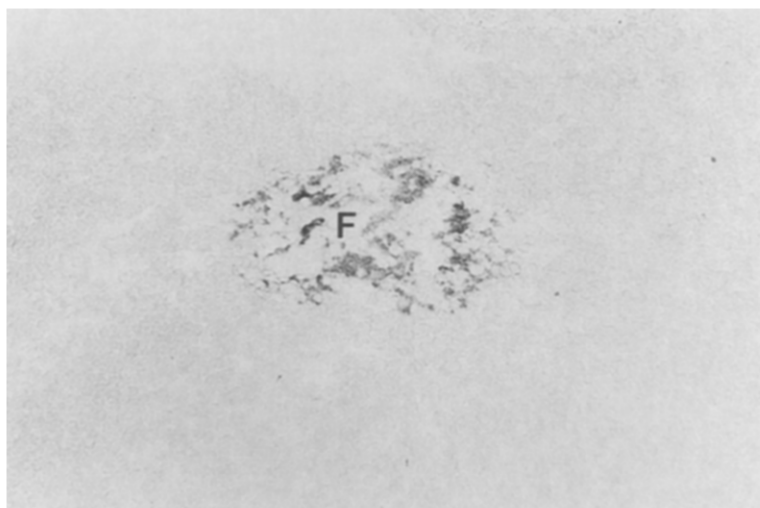


FIG. 4. Spleen from a patient vaccinated with Pneumovax. Demonstration of anti-PPS type 9V reactivity with FDCs, without preincubation with PPS ($\times 140$). F, Follicle center.

B-cell surface. Localization of NHS-incubated PPS was found in a meshwork pattern in the GC, consistent with FDC staining, and very weakly on mantle zone B cells. Marginal zone B cells were negative.

Anti-C3d antiserum

Sections were stained as in the normal procedure with NHS-incubated PPS, but with anti-C3d antiserum instead of anti-PPS antiserum: GCs, the mantle zone, and the marginal zone showed positivity; PALS and red pulp were negative. This staining pattern was identical to the NHS-PPS staining pattern. Performing the staining procedure with IHS preincubation of PPS or with fresh serum and omission of PPS antigens, followed by anti-C3d antiserum, showed less positivity in the GCs. Mantle zones, marginal zones, PALS, and red pulp were negative.

DISCUSSION

The results of this study show that the localization patterns of the different types of PPS are very similar. This indicates that the initial site of localization of the antigens does not depend on the type of PPS. Although it is known that a common component, C-polysaccharide, may contaminate specific PPS preparations, this C-polysaccharide is not recognized by the type-specific anti-PPS antisera. Therefore this contaminant cannot be responsible for this similarity of localization of different PPS types. The localization of the antigens

does not appear to be influenced by systemic diseases. The results of staining procedures with complement containing NHS show positivity in both the marginal zone and the mantle zone. The marginal zone stains more strongly than the mantle zone. Further, in the GC a dendritic pattern is seen, compatible with FDC staining, confirmed by double labeling for CD21. GC B cells are difficult to evaluate but seem negative, as confirmed by double labeling for CD20. This pattern is identical to the pattern observed for CD21.^{18, 19} These results confirm and extend our preliminary results from a study of the blocking capacity of different anti-CD21 antibodies in relation to NHS-incubated PPS.¹⁹

Spleens of preoperatively vaccinated versus non-vaccinated patients produced different results after a staining procedure with IHS. The positive GCs observed in sections of spleens of vaccinated subjects suggest that antigens and/or antibodies were present in the spleen before staining. After a successful immune response to the antigens in the PPS vaccine, as expected, no antigens were found at this time after vaccination in the mantle zone or the marginal zone. The restricted GC localization of PPS is based on the retention of PPS antigen/anti-PPS-antibody complexes by FDCs in the follicle centers as always occurs after a successful humoral immune response. This localization is relevant for affinity maturation and generation of B-cell memory.²⁰ This localization of PPS from the Pneumovax vaccine as a part of an immune com-

plex lasts much longer than the binding of pure PPS antigens in the mantle zone or the marginal zone.²¹ Spleens of patients who had not been vaccinated, and in whom therefore no immune response to the antigens in the PPS vaccine had occurred, showed only negative GCs.

In spleen sections of the vaccinated patients, positivity is also seen when PPS types (e.g., PPS 6A), which are not present in the vaccine (Pneumovax), are used. This may be due to extensive immunologic cross-reactivity, observed among pneumococcal capsular polysaccharides.⁷ For some PPS types present in the vaccine (e.g., PPS 12F), the follicle centers were negative after the staining procedure with IHS. The most likely explanation is that the vaccination did not produce an optimal immune response to this antigen, with consequent low or absent localization of PPS immune complexes in the GCs.

PPS 10A and less explicit PPS 4 show very weak or even negative staining. Several explanations are possible. One explanation is that the anti-PPS antiserum mainly recognizes epitopes at or near the binding site of C3 components to the PPS. It is also possible that these types of PPS do not bind to the C3 complement fragment as well as the other types do. Further, the antiserum could be of less quality than the other antisera. Moreover, for the detection of different types of PPS in human serum with an ELISA system, a two to three times higher threshold value is found for PPS type 10A in contrast to other types of PPS.²²

In this study we found three strong indications that C3d is the most important complement component in binding to PPS and subsequent binding of the complex to CD21.¹¹ First, sections stained with NHS showed a localization pattern identical to the staining patterns of sections stained with anti-CD21/CR2 mAbs. This pattern is clearly different from the pattern observed with antibodies directed against the C3b receptor (CD35). The latter shows, besides staining of marginal zone, mantle zone, and FDCs, strong staining of red pulp granulocytes.¹⁷ The second indication is that PPS staining procedures with NHS show positivity, whereas staining procedures with IHS showed negative results (excluding the sections of spleens of preoperatively vaccinated patients). The third indication is that spleen sections incubated with anti-C3d antiserum only show positive FDC staining, suggesting the presence of C3d epitopes, presumably as a component of immune complexes localized at FDCs. Performing the same protocol with sections preincubated with a PPS type and

NHS not only showed FDC positivity in the follicle centers but also in mantle and marginal zone B cells. Clearly, a component of complement, presumably C3d, is at least a component of the newly formed complexes localizing at the surface of the marginal zone (and less intensely, the mantle zone) B cells.

The above findings are corroborated by the results in newborn and infant spleens. Marginal zone cells in infants showed no or faint staining, either for PPS or for CD21. In all spleens the pattern of PPS localization was very similar to the obtained CD21 pattern, and different from CD35, which strongly stained mantle and marginal zone cells, and in addition, granulocytes. PPS incubated with IHS showed no reactivity in any of the newborn or infant spleens.

It is clearly shown that without CD21 at the B-cell surface, no localization of PPS is observed. Although in the normal immune response complement component C3b is often involved in formation of immune complexes, this seems of far less importance in localization of PPS. PPSs preincubated with fresh serum, which can be considered to contain all complement fragments, do localize at cells with CD21 and CD35 (C3b receptor) at the cell surface, but not at CD35+, CD21- cells as granulocytes and infant spleen marginal zone cells. Although the anti-C3d antiserum also recognizes epitopes on C3, C3b, and iC3b, the fact that cells with receptors for these complement fragments showed no PPS localization suggests that these fragments are less or not at all involved in complex formation with PPS. Fc receptor-positive cells, abundantly present in the spleen, do not show localization of incubated PPS antigens. This suggests that immunoglobulins are not a part of the localized PPS complex.

Our study shows that in human beings, PPSs, which can be considered natural TI-2 antigens, localize exclusively in areas with cells known to have (high) CD21 density at the cell surface. This localization is dependent on the presence of fresh serum factors including complement components.

Our findings suggest that the initiation of a primary humoral response to the most antigenic part of pneumococci, the capsular polysaccharide, is dependent on a mechanism different from most other responses. A relationship between the presence of C3 and the ability to mount an adequate antipneumococcal response has been reported.²³ According to the results of our study, it is likely that PPSs are not bound to C3b, but to C3d. In contrast to C3b binding, this complex is formed without the need for

specific immunoglobulin, which apparently is not a part of the complex. Such a mechanism has the advantage that even at first contact with a particular antigen, close contact is possible between the antigen-C3d complex and B cells with high CD21 (and IgM) density, which are particularly well equipped for a rapid activation and response.^{3,5,24,25} This activation is enabled by extensive cross-linking of CD21 and IgM on the B-cell surface. In a secondary response, localization of PPS-C3d complexes on FDCs can be expected to allow rapid isotype switching and perhaps affinity maturation^{20,26} of PPS-specific B (memory) cells, even in the absence of specific immunoglobulin. In this way it may be explained that in immunization procedures with PPSs, often in a first screening, IgM and also IgG antibodies are found.⁷

In conclusion, the results of this study contribute to an explanation of the dependency of the initiation of a primary humoral response to TI-2 antigens on the presence of a functionally intact spleen. The spleen is the only lymphoid organ with a specific B-cell compartment with high CD21 density at the cell surface. The anatomic context of this compartment, the marginal zone, allows close contact of antigen, bound to C3d, with these strongly CD21+ B cells.^{1,8,17} A secondary response, in normal immunophysiology, mainly dependent on the presence of GCs, is possible in all secondary lymphoid tissues. Our findings suggest that this secondary response may occur very rapidly after the induction of a primary response, because the PPS antigens can localize at FDCs, even if specific immunoglobulin is not (yet) present.

We thank Mr. Hilbrand Wieringa and Mr. Siep Noorman for their help in the preparation of the microphotographs.

REFERENCES

1. Timens W, Boes A, Rozeboom-Uiterwijk T, Poppema S. Immaturity of the human splenic marginal zone in infancy: possible contribution to the deficient infant immune response. *J Immunol* 1989;143:3200-6.
2. Van den Eertwegh AJM, Laman JD, Schellekens MM, Boersma WJA, Claassen E. Complement-mediated follicular localization of T-independent type-2 antigens: the role of marginal zone macrophages revisited. *Eur J Immunol* 1992;22:719-26.
3. Timens W, Boes A, Poppema S. Human marginal zone B cells are not an activated B cell subset: strong expression of CD21 as a putative mediator for rapid B-cell activation. *Eur J Immunol* 1989;19:2163-6.
4. MacLennan ICM, Liu Y-J. Marginal zone B cells respond both to polysaccharide antigens and protein antigens. *Res Immunol* 1991;142:346-51.
5. Timens W. The human spleen and the immune system: not just another lymphoid organ. *Res Immunol* 1991;142:316-20.
6. Brown AR. Immunological functions of splenic B-lymphocytes. *Crit Rev Immunol* 1992;11:395-417.
7. Lee C-J, Banks SD, Li JP. Virulence, immunity, and vaccine related to *Streptococcus pneumoniae*. *CRC Crit Rev Microbiol* 1991;18:89-114.
8. Timens W, Leemans R. Splenic autotransplantation and the immune system: adequate testing required for evaluation of effect. *Ann Surgery* 1992;215:256-60.
9. Cooper NR, Moore MD, Nemerow GR. Immunobiology of CR2, the B lymphocyte receptor for Epstein-Barr virus and the C3d complement fragment. *Annu Rev Immunol* 1988;6:85-113.
10. Ahearn JM, Fearon DT. Structure and function of the complement receptors, CR1 (CD35) and CR2 (CD21). *Adv Immunol* 1989;46:183-219.
11. Griffioen AW, Rijkers GT, Janssens-Korpela P, Zegers BJM. Pneumococcal polysaccharides complexed with C3d bind to human B lymphocytes via complement receptor type 2. *Infect Immun* 1991;59:1839-45.
12. Griffioen AW, Sanders L, Rijkers GT, Zegers BJM. Cell biology of B lymphocyte activation by polysaccharides. *J Infect Dis* 1992;165(suppl 1):S71-S73.
13. Griffioen AW, Franklin SW, Zegers BJM, Rijkers GT. Expression and functional characteristics of the complement receptor type 2 on adult and neonatal B lymphocytes. *Clin Immunol Immunopathol* 1993;69:1-8.
14. Laman JD, Van den Eertwegh AJM, Claassen E, Van Rooijen N. Cell-cell interactions: in situ studies of splenic humoral immune responses. In: Fornusek L, Vetvicka V, eds. *Immune system accessory cells*. Boca Raton, Florida: CRC Press, 1992:201-31.
15. Knapp W, Dörken B, Gilks WR, et al. *Leucocyte typing IV. White cell differentiation antigens*. Oxford: Oxford University Press, 1989.
16. Marquart HV, Svehaug S-E, Leslie RGQ. CR2 is the primary acceptor site for C3 during alternative pathway activation of complement on human peripheral B lymphocytes. *J Immunol* 1994;153:307-15.
17. Timens W, Poppema S. Lymphocyte compartments in human spleen. An immunohistologic study in normal spleens and non-involved spleens in Hodgkin's disease. *Am J Pathol* 1985;120:443-54.
18. Timens W. B-cell antigens: CD21 Workshop panel report. In: Schlossman SF, Boumsell L, Gilks W, et al., eds. *Leucocyte typing V. White cell differentiation antigens*. Oxford: Oxford University Press, 1994:516-8.
19. Timens W, Harms G, Peset-Llopis MJ, Hardonk M. Pneumococcal polysaccharides localize on splenic marginal zone B-cells and follicular dendritic cells, together with complement fragment C3d, without relation to known CD21 epitopes. In: Schlossman SF, Boumsell L, Gilks W, et al., eds. *Leucocyte typing V. White cell differentiation antigens*. Oxford: Oxford University Press, 1994:519-20.
20. Kroese FGM, Timens W, Nieuwenhuis P. Germinal center reaction and B lymphocytes: morphology and function. In: Grundmann E, Vollmer E, eds. *Current topics in pathology vol. 84/1. Reaction patterns of the lymph node 1: cell types and functions*. Berlin: Springer-Verlag, 1990:103-48.

21. Liu Y-J, Johnson GD, Gordon J, MacLennan ICM. Germinal centres in T-cell-dependent antibody responses. *Immunol Today* 1992;13:17-21.
22. Schaffner A, Michel-Harder C, Yeginsoy S. Detection of capsular polysaccharide in serum for the diagnosis of pneumococcal pneumonia: clinical and experimental evaluation. *J Infect Dis* 1991;163:1094-102.
23. Hazlewood MA, Kumararatne DS, Webster ADB, Goodall M, Bird P, Daha M. An association between homozygous C3 deficiency and low levels of anti-pneumococcal capsular polysaccharide antibodies. *Clin Exp Immunol* 1992;87:404-9.
24. Boyd AW, Anderson KC, Freedman AS, et al. Studies of in vitro activation and differentiation of B cells: phenotypic and functional characterization of the B cell population responding to anti-Ig antibody. *J Immunol* 1985;134:1516-23.
25. Carter RH, Spycher MO, Ng YC, Hoffman R, Fearon DT. Synergistic interaction between complement receptor type 2 and membrane IgM on B lymphocytes. *J Immunol* 1988;141:457-63.
26. Kraal G, Weissman IL, Butcher EC. Germinal center B-cells: antigen specificity and changes in heavy chain expression. *Nature* 1982;298:377-9.