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Antibody-forming cell induction during an early phase of germinal centre development and its delay with ageing

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SUMMARY

The present study was initiated to determine if an early phase of germinal centre (GC) development is associated with the generation of antibody-forming cells (AFC). Germinal centres in draining lymph nodes from immune mice were examined histochemically after secondary immunization for the presence of AFC at both the light and electron microscopic levels. Additionally, peanut agglutinin (PNA) high (^{Hi}) GC B cells were isolated, placed in cell culture and specific antibody production was monitored at successive intervals. Electron microscopy showed that plasma cells in all stages of differentiation were present within GC at 3–5 days and to a lesser extent at 7 days following antigenic challenge. Furthermore, PNA^{Hi} GC B cells obtained between Days 3 and 5 spontaneously produced specific IgG when placed in culture. Germinal centre B cells isolated either before or after this period did not produce antibody without the addition of T-cell cytokines. Induction of AFC in GC occurred at the time when GC B cells acquire follicular dendritic cell (FDC)-derived, immune complex-coated bodies (iccosomes) and process and present this antigen to helper T cells. This suggested a causal relationship between iccosome release and AFC induction. Support for this was obtained by examination of AFC induction in aged mice where iccosome release has not been observed. Peanut agglutinin-positive GC B cells isolated from aged mice on Days 3–5 after antigen challenge failed to spontaneously produce specific antibody. Collectively, these data show that GC development 3–5 days after booster immunization results in AFC generation and suggests a role for FDC iccosomes in their induction.

INTRODUCTION

Germinal centres (GC) are sites of rapidly proliferating B lymphocytes which are found in secondary lymphoid tissue in association with antigen-bearing follicular dendritic cells (FDC), T cells and tingible body macrophages. Interactions between these cells during GC formation in immune mice is a major interest in our laboratories. Analysis of the kinetics of events in the GC reaction has focused on the 2-week period after booster immunization (Szakal, Kosco & Tew, 1988a; Szakal *et al.*, 1988b; Kosco, Szakal & Tew, 1988; Smith *et al.*, 1988; Szakal, Kosco & Tew, 1989a; Szakal *et al.*, 1989b; Tew, Kosco & Szakal, 1989).

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Abbreviations: AFC, antibody-forming cell(s); Con A, concanavalin A; DAB, diaminobenzidine; EM, electron microscopy; FBS, fetal bovine serum; FDC, follicular dendritic cell(s); GC, germinal centre(s); HBSS, Hanks' buffered salt solution; HRP, horseradish peroxidase; HSA, human serum albumin; iccosomes, immune complex-coated bodies; OVA, ovalbumin; PBS, phosphate-buffered saline; PNA, peanut agglutination.

In a recent study we followed the kinetics of GC development by use of peanut agglutinin (PNA) as a marker for GC B cells (Coico, Bhogal & Thorbecke, 1983; Szakal *et al.*, 1989b). Preexisting GC dissociate during the first 3 days after challenge (Hanna, Congdon & Wust, 1966; Szakal *et al.*, 1989b) and new GC begin to develop by Day 3, with maximum GC size achieved by Day 10 (Szakal *et al.*, 1989b). The present study was prompted by the observation that in lymph nodes as late as Day 5 post-antigen challenge, PNA-positive cells appear to be migrating from the GC towards the medullary cords, a site of plasma cell differentiation. This migration occurred just following the time when FDC release immune complex-coated bodies (iccosomes) (Szakal *et al.*, 1988a). Germinal centre B cells endocytose iccosomes and process the antigen, which can be presented to T cells (Kosco *et al.*, 1988). The finding of PNA-positive cells apparently migrating into medullary cords coupled with the observation that plasma cell development could be seen within the GC (Sordat *et al.*, 1970; Terashima *et al.*, 1977) led us to wonder if GC served as an induction site for AFC.

Our hypothesis is that during an early phase of the germinal centre reaction a subpopulation of GC B cells receives appropriate signals to differentiate into antibody-forming cells

(AFC). As this dynamic process continues, renewal and expansion of the B-memory cell compartment occurs. To test this hypothesis, lymph node sections were evaluated at various times after secondary immunization at the light and electron microscopic levels using immunohistochemistry. In addition, at various times after immunization PNA^{Hi} GC B cells were isolated, placed in cell culture and specific antibody synthesis was monitored. The results reported here indicate that AFC are induced at an early stage of the GC reaction. At a later stage, GC B cells appear to represent B-memory cells and no longer make specific antibody *in vivo* or *in vitro* in the absence of further stimulation.

MATERIALS AND METHODS

Animals

Female C3H/HeJ, BALB/cJ or BALB/cByJ mice, aged 6–8 weeks, were obtained from Jackson Laboratories, Bar Harbor, ME. Aged mice (>23 months of age) and young controls were obtained from the National Institute of Aging breeding colony at Charles River Laboratories, Wilmington, MA. Animals were housed in standard-size shoe-box cages and given food and water ad libitum.

Immunization

The histochemically detectable antigen horseradish peroxidase (HRP; Type VI, Sigma Chemical Co., St Louis, MO), and human serum albumin (HSA; Sigma) or ovalbumin (OVA; Sigma) were used for immunization. Mice were primed by injection of 100 μg of antigen in complete Freund's adjuvant behind the neck and challenged 2 weeks later with 100 μg of the same antigen preparation distributed among the four footpads and 100 μg behind the neck. Twenty-one days later, the mice were again injected behind the neck and in the footpads with antigen (approximately 10 μg/injection site) dissolved in Dulbecco's phosphate-buffered saline (PBS). For localization of PNA-positive GC, mice were passively immunized as described by Szakal *et al.* (1988b). Briefly, mice were injected with 0·5 ml rabbit anti-HRP followed by injection of 8 μg HRP in the footpads 24 hr later.

Immunohistochemistry

Germinal centres and HRP-specific plasma cells were visualized by peroxidase histochemistry. Following immunization, mice received intracardiac perfusion with 30 ml heparinized, Ringer's solution (37°) followed by paraformaldehyde (1%)/glutaraldehyde (0·9%) fixative (4°, pH 7·4). Subsequently the lymph nodes were excised, immersed in the same fixative for 4–6 hr and then washed in cacodylate buffer (0·1 M, pH 7·4) for 24 hr.

Localization of GC was achieved by utilizing the ability of GC B cells to bind high levels of PNA (Coico *et al.*, 1983). Fifty-micrometre thick vibratome sections of lymph nodes were incubated in PBS containing 125 μg/ml PNA-HRP conjugate (L-7759; Sigma) at 4° for 24 hr. The sections were washed for 24 hr in Tris-HCl buffer (0·05 M, pH 7·6) at 4°. Peroxidase activity was developed by incubating the sections in diaminobenzidine hydrochloride (DAB; 0·5 mg/ml) in Tris-HCl buffer containing 0·01% H_2O_2, plus 10 mM 3-amino-1,2,4-triazole (A4383, Sigma) (Herzog & Miller, 1972) to inhibit endogenous peroxidase activity and 0·1% crystalline bovine serum albumin for 10 min at ambient temperature. No GC were labelled in control sections without preincubation in PNA-HRP or after incubation in a DAB solution without the substrate H_2O_2.

For ultrastructural evaluation, 3, 4 and 7 days after footpad challenge with HRP, the draining lymph nodes were obtained and processed as above. Germinal centres, identified by the localization of HRP on follicular dendritic cells, were cut out, osmicated and processed for electron microscopy (EM) according to the technique reported elsewhere (Szakal, Holmes & Tew, 1983). To study the differentiation of specific antibody-forming cells (AFC) from GC B cells, an immunoperoxidase technique developed in our laboratories was applied (Donaldson *et al.*, 1986). This technique allows the localization of HRP-specific antibodies synthesized within the cytoplasm of developing AFC and fully mature cells. Briefly, sections are preincubated with methanol and 0·02% H_2O_2 for 1 hr to eliminate endogenous peroxidase activity. Consequently, the methanol treatment effects the plasma membranes such that in subsequent steps, molecules can diffuse easily in and out of cells. To localize specific AFC, the sections are then incubated overnight in buffer containing 20% FBS plus either 100 μg/ml HRP or 125 μg/ml HRP conjugated to PNA. Using HRP-PNA allows the simultaneous visualization of both PNA-positive areas and HRP-specific AFC. The sections are washed for 24 hr and the peroxidase label developed using DAB and H_2O_2.

Isolation and enrichment of GC B cells

The PNA^{Hi} GC B cells were isolated from immune mouse lymph nodes as described previously (Kosco *et al.*, 1988). Briefly, lymph node cell suspensions were prepared by enzymatic dissociation for 1 hr at 37° in Hanks' balanced salt solution (HBSS) containing collagenase (CLS 4; Worthington Biochemical Corporation, Freehold, NJ), protease (no. P6141; Sigma) and DNase (no. D0876; Sigma). After isolating the low density B-cell fraction (1·060–1·065 g/ml) on a continuous Percoll (no. 17-0891-01; Pharmacia, Piscataway, NJ) density gradient, the cells were washed, and a 5-ml cell suspension (containing 3×10^7 cells/ml in HBSS containing 2% FBS; Hyclone, Logan, UT) was then plated to PNA (no. L0881; Sigma)-coated petri-dishes (no. 1005; Falcon, Becton-Dickinson and Co., Lincoln Park, NJ). Petri-dishes were coated by overnight incubation with 125 μg of PNA in bicarbonate buffer (pH 9·6). The plates were then incubated for 1 hr at 4°. Non-binding cells were removed from the plates by gentle washing with HBSS. Subsequently, PNA-binding cells were eluted by incubation with 0·2 M galactose in HBSS containing 2% FBS for 45 min at 4°. The resulting cell suspension was then collected and washed three times prior to use.

Determination of specific antibody production by GC cells

Peanut $agglutinin^{Hi}$ GC B cells were obtained at various times after secondary immunization (i.e. Day 1, Day 3, etc.) and resuspended at 1×10^6 cells per ml DMEM (high glucose) containing 10% FBS, L-glutamine (2 mM), sodium pyruvate (1 mM), HEPES (25 mM) and gentamicin (50 μg/ml) (i.e. cell culture medium). One millilitre of cells was added per well of a 24-well culture plate and then incubated at 37°, 5% CO_2. Triplicate wells were harvested at various times and supernatant fluid collected and assayed for specific IgG using a solid-phase RIA.

RIA for specific antibody

The specific antibody content (anti-HRP or anti-OVA) of each tissue culture supernatant fluid sample was determined using a

double-antibody technique modified from that described earlier (Tew *et al.*, 1985). Briefly, antigen was diluted in carbonate buffer (0·05 M $NaHCO_3$; 0·02 M Na_2CO_3, pH 9·6) at a concentration of 1 mg/ml and adsorbed to the surface of polystyrene flat-bottomed culture wells (Immulon 1 Removawells, Dynatech, Chantilly, VA) by incubation at room temperature for a minimum of 2 hr. The wells were then immersed and decanted 20 times in large quantities of tap water and allowed to air dry. A pooled serum sample (referred to hereafter as the antibody standard) of mouse anti-HRP or anti-OVA was serially diluted in a PBS/milk solution (10 g powdered milk/100 ml PBS) at dilutions of 1:50 out to 1:102,400. The antibody standards contained 146 μg/ml of anti-HRP or 3500 μg/ml anti-OVA, respectively, as determined by quantitative precipitation. Two-hundred microlitres of each dilution of culture fluid or antibody standard were transferred into antigen-coated and uncoated wells (to determine non-specific binding) and allowed to incubate overnight at 4°. The wells were then washed in tap water as described above. Goat $F(ab')_2$ anti-mouse IgG (Fc specific) (Jackson ImmunoResearch Laboratories Inc., Avondale, PA) was labelled with ^{125}I (Amersham, Arlington Heights, IL) to a specific activity of approximately 30 μCi/μg (1·11 MBq/μg) antibody. The radiolabelled antibody was diluted in the PBS/milk solution such that 200 μl of solution yielded 200,000–300,000 c.p.m. Two-hundred microlitres of this solution were then added to each of the test wells. Following overnight incubation at 4°, the wells were washed as described above, air dried, and counted for radioactivity. Non-specific binding of tissue culture supernatant fluid to the plastic wells was not observed. The results were expressed as ng/ml of specific antibody based on a comparison between the sample c.p.m. and c.p.m. of the appropriate antibody standard.

RESULTS

Localization of GC by peroxidase-conjugated PNA

Evaluation by light microscopy showed the presence of clearly defined PNA-positive GC in the cortex of popliteal lymph nodes of immune mice 5 days after challenge with antigen in the hind footpads (Fig. 1a, b). As new GC were developing a subpopulation of PNA-positive cells appeared to be leaving these GC (Fig. 1b, arrowheads). Their distribution was within the medullary cords, suggesting a migration of PNA-positive GC cells into the medulla (Fig. 1b), where plasma cells accumulate at this time. To determine if these PNA-positive cells were differentiating into plasma cells, GC were then examined for the presence of specific antibody-forming cells.

Immunoperoxidase detection of plasma cell differentiation in GC and medullary cords

In agreement with previous ultrastructural descriptions of specific antibody-producing plasma cell differentiation (Leduc, Avrameas & Bouteille, 1968; Terashima *et al*, 1977), HRP was found localized in GC cells of the plasmacytic series, in the cisterna of the nuclear envelope of plasmablasts and primarily in the cisternae of the rough endoplasmic reticulum of proplasmacytes (Szakal *et al.*, 1989a). Occasional, fully differentiated HRP-specific plasma cells were also observed in GC. These HRP-specific cells were distributed in close proximity to the antigen-retaining dendritic processes of FDC as well as deeper in the dark zone of GC. These surveys showed an increase in the frequency of differentiating HRP-specific plasma cells from Day 3 to Day 4 and a reduction in their numbers in GC by Day 7. The observations that fewer plasma cells than HRP-specific proplasmacytes were present in GC suggested that completion of maturation occurs outside GC. To examine this further, sections of lymph nodes were preincubated with methanol followed by PNA-HRP to label both the PNA^{Hi} cells in the GC and the HRP-specific plasma cells. Using this technique, cells in the latter stages of plasma cell differentiation can be identified at the light microscopic level (Donaldson *et al.*, 1986). At Day 7 (Fig. 1c–f), HRP-specific plasma cells (arrows) were observed in trails extending from within PNA-positive GC through medullary cords (arrowheads) towards the deeper parts of the medulla. These trails further support the hypothesis that plasma cells tend to leave the GC and complete maturation outside the GC.

In vitro antibody production by PNA-positive GC cells

Although plasma cells are PNA negative (Kraal, Weissman & Butcher, 1982), we reasoned that the PNA marker may be on differentiating GC immunoblasts or plasmablasts shortly after transition from the immunoblast stage. If PNA-positive cells spontaneously secrete antibody, it would indicate that cells within the GC receive the necessary signals for differentiation and production of antibody. To test this, PNA^{Hi} cells were isolated at successive times after secondary immunization, placed in culture for 1 week, and their ability to produce specific IgG was monitored. The results showed (Fig. 2) that GC B cells isolated at Day 4 after challenge spontaneously produced large amounts of specific antibody, and most was produced during the first 24 hr, indicating that AFC were rapidly maturing *in vitro*. In contrast, cells isolated at Day 7 and at Day 12 produced very little specific antibody spontaneously.

Table 1. Effect of cytokines on spontaneous antibody production by PNA^{Hi} GC cells*

Time after Ag challenge‡	Time in culture	Medium only (anti-HRP IgG ng/ml)	Plus Con A sup (anti-HRP IgG ng/ml)
1 day	24 hr	3±0·2	5±0·1
	7 days	6±1·3	56±5·5
3 days	24 hr	183±6·3	189±2·3†
	7 days	314±1·7	346±7·8†
7 days	24 hr	4±0·1	13±0·8
	7 days	9±0·8	87±2·5

* PNA^{Hi} GC B cells were isolated at various times following booster immunization and incubated at 10^6 cells/well in medium alone or in medium plus Con A supernatant (20%). Culture supernatant was collected from triplicate wells at 24 hr or 7 days and specific IgG production was determined using an RIA.

† Although not apparent in this experiment, the addition of Con A supernatant fluid caused statistically significant augmentation of antibody production by Day 3 PNA^{Hi} GC cells in some experiments.

‡ Ag, antigen.

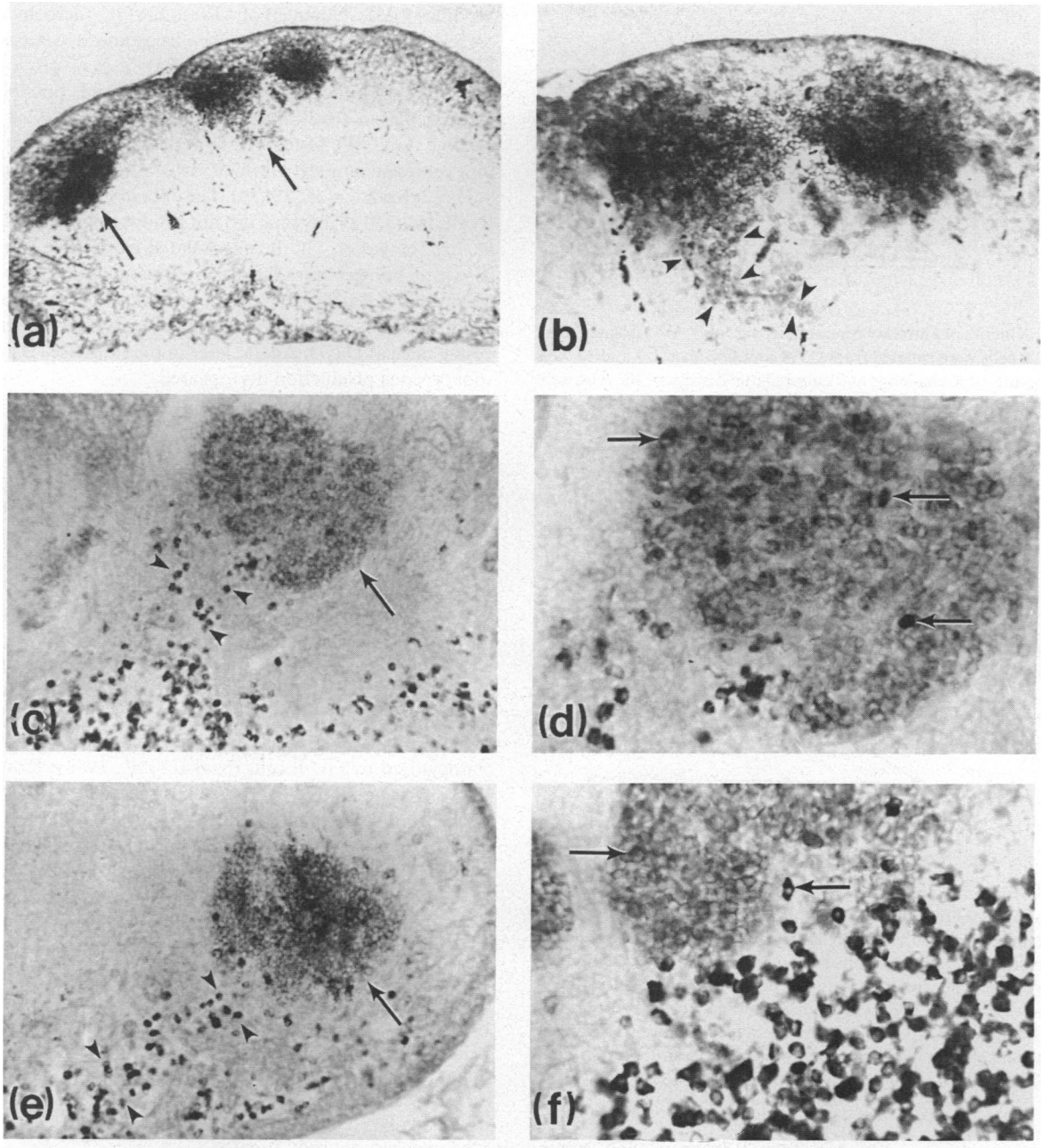

Figure 1. Light micrographs demonstrating the distribution of PNA-positive GC B cells and HRP-binding plasma cells in lymph node sections from passively immunized mice. In (a) and (b), the vibratome sections were incubated in HRP-PNA to localize GC. In (c)–(f), the sections were preincubated in methanol followed by HRP-PNA in order to label the GC and intracytoplasmic anti-HRP. (a) Shows a typical lymph node section (× 40) 5 days after antigen challenge. Note the specific staining of PNA positive GC (arrows). (b) Shows a higher magnification (× 100) of the same lymph node section with a distinct trail of PNA-positive cells (arrowheads) from the GC into the medulla. In (c) and (e) (× 100) note that HRP-specific plasma cells (arrowheads) forming a continuum between the GC and the medulla. Specific plasma cells can be seen in (d) and (f) (× 195 and 190, respectively) within GC as well as in the medulla (f).

We reasoned that at Day 7 and Day 12, PNA^{Hi} GC B cells had been stimulated by antigen and may be able to differentiate if given appropriate T-cell factors. Therefore GC B cells were cultured as before but in either the presence or absence of a source of T-cell cytokines [Concanavalin A (Con A) stimulated, rat spleen cell supernatant which had been absorbed to remove residual Con A]. The data shows (Table 1) that cells isolated at Day 3 produced significant levels of specific IgG, whereas cells isolated at either Day 1 or Day 7 of the reaction did not. The addition of Con A supernatant to the cells isolated on Day 3 of the reaction was not required for antibody production, although in some experiments it did cause augmentation. In contrast, significant antibody production was not achieved without the addition of Con A supernatant to cultures of GC cells isolated on Day 1 or 7. The addition of T-cell cytokines to these cultures resulted in a nine-fold augmentation of anti-HRP production.

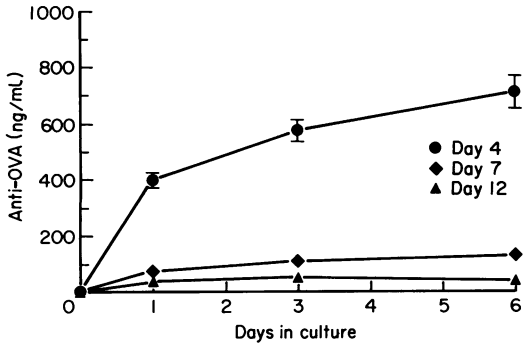

Figure 2. Kinetics of antibody production by PNA^{Hi} AFC isolated from GC. GC B cells were isolated from OVA-immune mice 4, 7 and 12 days following antigenic challenge and cultured at a density of 10^6 cells/well. Culture supernatant was collected from triplicate wells after 1, 3 and 6 days and specific IgG production was determined using a solid-phase RIA. Only PNA^{Hi} GC cells isolated on Day 4 produced a significant antibody response spontaneously. Note that most of the antibody was produced during the first day in culture, suggesting a rapid differentiation into the plasma cell compartment.

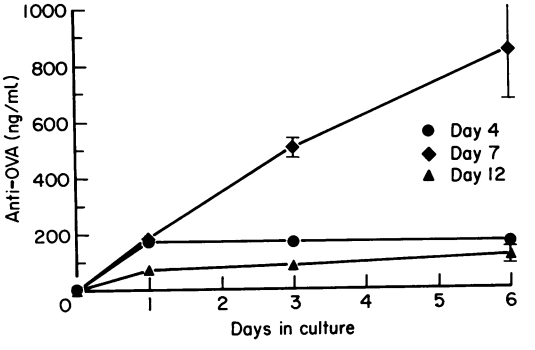

Figure 3. Kinetics of antibody production by PNA^{Hi} AFC isolated from GC of aged (>23 months) mice. GC B cells were isolated from OVA-immune mice, cultured and their antibody production determined as described in Fig. 2. Spontaneous antibody was not produced at Day 4 when iccosome release in young mice was ongoing. Surprisingly, significant specific antibody was produced spontaneously at Day 7. This time correlated with a decrease in the overall size of the FDC antigen-retaining reticulum in these aged mice. Note that the antibody produced at Day 7 on a cell to cell basis is comparable to that seen in young mice.

Since the development of AFC in the GC microenvironment correlated well with the time when iccosomes are released from FDCs (Day 3; Szakal *et al.*, 1988a), it was proposed that iccosomes (containing specific antigen and possibly other factors) play a functional role in stimulating GC B cells to become AFC. We tested this hypothesis using aged mice since they represent a model in which no iccosome release is observed at Day 3 (Szakal *et al.*, 1988b). The data in Fig. 3 shows that GC B cells isolated at this time did not produce significant antibody *in vitro*. However, GC B cells isolated from these aged mice 7 days after booster immunization spontaneously produced specific antibody. Furthermore, on a cell to cell basis this antibody production was comparable to that seen in cells obtained from young mice at Day 3. Again, later in the response (Day 12) this spontaneous production disappeared.

DISCUSSION

The present study revealed that specific AFC appear within the GC microenvironment 3–5 days following booster immunization. Specific plasma cells were observed in all stages of differentiation within GC. Furthermore, PNA^{Hi} cells obtained 3–5 days after booster immunization spontaneously produced specific IgG when placed in culture. Such cells isolated either before or after this period did not produce antibody without addition of T-cell cytokines. The necessary conditions for AFC induction are apparently present within GC during a specific interval following antigen challenge. This interval coincides with the time when FDC release iccosomes that are known to be endocytosed by GC B cells (Szakal *et al.*, 1988a; Kosco *et al.*, 1988). We suggest that B cells process and present this iccosome-derived antigen to helper T cells in the GC and that T cells provide these specific B cells with the factors necessary for them to differentiate into plasma cells. When iccosome release by FDC ceases, the B cells may continue to proliferate and form large numbers of specific memory cells but no longer receive the T-cell factors needed to differentiate into AFC.

Important events at different stages of the GC reaction are summarized in Table 2. During the early phase, 1–2 days following booster immunization, pre-existing GC dissociate prior to new GC formation (Hanna *et al.*, 1966; Szakal *et al.*, 1989b). Antigen in the form of immune complex is transported

Table 2. Summary of GC events during the secondary antibody response

Phase	GC status	FDC Ag* state	Ag endocytosis, processing and presentation by GC B cells	PNA-positive AFC in GC	PNA-positive memory cells in GC
Early (Days 1–2)	Dissociation of pre-existing GC	Ag trapped and retained by FDC	—	±	ND
Intermediate (Days 3–5)	Initiation of *de novo* GC	Iccosomes released	++++ (peaks at Day 5)	++++ (Day 5)	++
Late (Days 6–14)	Full development of GC	Ag 'shielded' for long-term retention	++ (minimal activity after Day 9)	±	++++ (Day 10)

ND, not determined.
* Ag, antigen.

into draining lymph nodes and is either eliminated by macrophages or trapped by non-phagocytic antigen-transporting cells (Szakal *et al.*, 1983). The transported antigen is seen on FDC within hours after immunization and the transport is completed within 1 day (Szakal *et al.*, 1983). PNA^{Hi} GC B cells isolated from the lymph node 1 day after booster immunization were not capable of spontaneous antibody production, indicating that the necessary signals for AFC differentiation had not been received *in vivo*. However, specific B cells were present as evidenced by the ability to induce specific antibody production by addition of a source of T-cell cytokines. The source of these specific B cells may be the pre-existing GC which are undergoing dissociation at this time.

In the 3–5 day period (intermediate phase), the GC microenvironment changes significantly. *De novo* GC formation begins around the FDC retained antigen (Szakal *et al.*, 1989b). Many of the FDC filiform dendritic processes become beaded, forming iccosomes (Szakal *et al.*, 1985), which are then dispersed within this area (Szakal *et al.*, 1988a). They are then endocytosed by GC B cells which process and present the antigen to T cells (Szakal *et al.*, 1988a; Kosco *et al.*, 1988). This antigen presentation reaches a peak about Day 5 (Kosco *et al.*, 1988). Recently, studies by Monfalcone *et al.* (1989) revealed that these GC cells are also capable of eliciting potent allogeneic and even syngeneic mixed lymphocyte reactions. As reported here, GC B cells isolated at this time spontaneously produced specific IgG and this was not significantly augmented by the addition of T-cell factors. It appears that the necessary signals for B-cell differentiation were present in GC during this interval.

In the late phase of the reaction, GC size increases reaching a maximum about Day 10 (Coico *et al.*, 1983; Szakal *et al.*, 1989b). The dendritic processes become tightly convoluted in such a manner that most of the retained immune complexes are shielded from the B cells (Szakal *et al.*, 1988a). Isolated GC B cells during this later phase did not produce antibody without the addition of cytokines. However, in agreement with Coico *et al.* (1983), adoptive transfer of these cells resulted in transfer of B-cell memory (G. F. Burton, unreported data).

These data indicate that a narrow window existed between Day 3 and Day 5 for induction of AFC within GC. Although GC are not a major source of antibody production, it appeared that a subpopulation of cells did receive differentiation signals in the GC and then emigrated to terminally differentiate elsewhere. The GC B cells isolated between Days 5 and 10 appeared to be committed to memory.

In studies with aged mice, Szakal *et al.* (1988b) demonstrated that antigen transport was defective and the number of FDC markedly decreased. In addition, no iccosome release was observed between Days 3 and 5. Instead, the amount of antigen retained by FDC decreased around Day 7, suggesting a delay in the release of the immunogen (Szakal *et al.*, 1988b). The fact that antibody production by GC cells isolated from aged mice was detected on Day 7 instead of Day 3 suggested that iccosomes (i.e. the release of immunogen) may provide a necessary signal for terminal differentiation of GC B cells.

It appears that the potential of GC B cells to either differentiate into plasma cells or to memory cells depends in part on the phase of the GC reaction. Clearly, most GC B cells represent B memory cells (Klaus *et al.*, 1980; Coico *et al.*, 1983) and the data reported here are not contradicting these findings. In fact, these GC B cells obtained from both young and old mice 10 days post-antigenic challenge have produced good memory responses in adoptive transfer experiments (G. F. Burton, unreported data). Nevertheless, it appears that during the Day 3–5 period of a secondary immune response the GC may be providing stimulation such that some B cells become AFC. This may be particularly important when small but physiologically relevant doses of antigen are used. In this case, where immunogen is limited, antigen in the FDC pathway represents a major source of stimulant (Tew *et al.*, 1989). In short, it seems that the GC represents not only a source of B-memory cells but also a site for AFC induction.

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