Proc. Natl. Acad. Sci. USA
Vol. 93, pp. 10222–10227, September 1996
Cell Biology

Rad51 expression and localization in B cells carrying out class switch recombination

(germinal center/immunoglobulin/lymphocyte/protein immunofluorescence/spleen)

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Contributed by Charles M. Radding, June 21, 1996

ABSTRACT **Rad51 is a highly conserved eukaryotic homolog of the prokaryotic recombination protein RecA, which has been shown to function in both recombinational repair of DNA damage and meiotic recombination in yeast. In primary murine B cells cultured with lipopolysaccharide (LPS) to stimulate heavy chain class switch recombination, Rad51 protein levels are dramatically induced. Immunofluorescent microscopy shows that anti-Rad51 antibodies stain foci that are localized within the nuclei of switching B cells. Immunohistochemical analysis of splenic sections shows that clusters of cells that stain brightly with anti-Rad51 antibodies are evident within several days after primary immunization and that Rad51 staining *in vivo* is confined to B cells that are switching from expression of IgM to IgG antibodies. Following switch recombination, B cells populate splenic germinal centers, where somatic hypermutation and clonal proliferation occur. Germinal center B cells are not stained by anti-Rad51 antibodies. Rad51 expression is therefore not coincident with somatic hypermutation, nor does Rad51 expression correlate simply with cell proliferation. These data suggest that Rad51, or a highly related member of the conserved RecA family, may function in class switch recombination.**

Immunoglobulin heavy chain class switch recombination joins a heavy chain variable (VDJ) region to a new downstream constant (C) region, deleting the DNA between (reviewed in refs. 1–3). Each C region removes antigen by a distinct mechanism, so switch recombination changes how an immunoglobulin molecule removes antigen without altering its specificity for antigen. Switch recombination involves switch (S) regions, repetitive G-rich regions of DNA, 2–8 kb in length, which are located in the intron upstream of each C region that undergoes switching. Circular molecules containing the deleted C region and flanking sequences can be isolated from cultures of switching B cells (4–6), suggesting that during recombination distant S regions form a synaptic complex which undergoes cleavage and religation to produce a chromosomal switch junction and an excised switch circle (see Fig. 1). Extracellular signals mediated by cytokines and lymphokines target switching to particular C regions, in a process that correlates temporally with production of noncoding transcripts from that region. However, the rules that govern switch recombination are not clear: recombination junctions do not appear to involve specific sequences or runs of homology in the recombining S regions (7). This distinguishes switching from other targeted and regulated recombination processes in both eukaryotic and prokaryotic cells, and it makes the mechanism of switch recombination of particular interest.

Identification of proteins involved in switch recombination is a critical step in understanding the mechanism. As switching occurs only in B cells, recombination must depend at least in part upon B cell-specific factors that recognize S region sequences or critical cis-regulatory elements (8–14). Given the dependence of V(D)J joining on ubiquitous proteins such as Ku, DNA protein kinase, and the *XRCC4* gene product (15–20), it seemed likely that some subset of the recombination proteins that are ubiquitous in all cell types might also participate in switch recombination.

One especially interesting candidate for a role in switch recombination is Rad51, the highly conserved eukaryotic homolog of the prokaryotic recombination protein RecA. In yeast, Rad51 functions in meiotic recombination and is implicated in recombination-dependent double-strand break repair (21–24). The mouse (25) and human (26) Rad51 homologs are 83% identical with the yeast protein, and mammalian Rad51 has been strongly correlated with meiotic recombination and DNA repair. While Rad51 transcripts and protein are present in all mammalian cell types examined thus far, the highest transcript levels are found in tissues active in recombination, including spleen, thymus, ovary, and testis (27, 28). In spermatocytes undergoing meiosis, Rad51 is enriched in the synaptonemal complexes, which join paired homologous chromosomes (29–31). In cultured cell lines, localization of Rad51 changes dramatically in response to DNA damage, when multiple foci of Rad51 form in the nucleus and stain vividly with anti-Rad51 antibodies (29).

We have asked if Rad51 might function in switch recombination by using anti-Rad51 antibodies (29) to assay Rad51 expression and to localize Rad51 by immunofluorescence microscopy. We have found that Rad51 expression is dramatically induced in activated primary B cells, and that the appearance of $Rad51^+$ foci in the nuclei of these cells correlates with the switch from expression of IgM to IgG antibodies. In splenic sections from immunized mice, clusters of $Rad51^+$ B cells are evident and localize to regions of the spleen where switch recombination is occurring. Our results suggest that Rad51 (or a highly related member of the conserved RecA family of proteins) may function in switch recombination.

MATERIALS AND METHODS

Primary Splenocyte Culture. Spleens from three or more 8- to 12-week old BALB/c mice (The Jackson Laboratory) were disaggregated, and cells were washed twice with RPMI 1640 medium (GIBCO) and then treated for 5 min with 0.144 M NH_4Cl/0.017 M Tris·HCl, pH 7.4, to lyse erythrocytes. Recovered lymphocytes were washed once with phosphate-buffered saline (PBS; 137 mM NaCl/2.7 mM KCl/8.1 mM

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Abbreviations: C, constant; DAB, 3,3′-diaminobenzidine; DAPI, 4′,6-diamidino-2-phenylindole; FITC, fluorescein isothiocyanate; LPS, lipopolysaccharide; NP, (4-hydroxy-3-nitrophenyl)acetyl; PNA, peanut agglutinin; S, switch.

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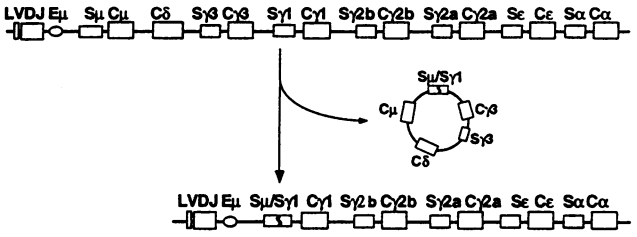

FIG. 1. Switch recombination. During recombination distant switch regions form a synaptic complex which undergoes cleavage and religation to produce a chromosomal switch junction and an excised switch circle. Switching from μ to γ1 is shown.

Na_2HPO_4/1.5 mM KH_2PO_4, pH 7.4) and cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 20 μM 2-mercaptoethanol, 100 units/ml penicillin, 100 μg/ml streptomycin, and 40 μg/ml lipopolysaccharide (LPS; from *Escherichia coli* O26:B6, Sigma).

Anti-Rad51 Antibody and Immunoblotting. Rabbit polyclonal anti-human Rad51 antibody was generated by immunization with purified recombinant human Rad51 protein (29). For immunoblots, cells were lysed in 50 mM Tris·HCl, pH 8.0/150 mM NaCl containing 100 μg/ml phenylmethylsulfonyl fluoride (PMSF), 1 μg/ml aprotinin, and 1% Triton X-100 for 30 min on ice and microcentrifuged to remove debris, protein concentrations were determined by Bradford assay (Bio-Rad), and 10 μg of protein was resolved by SDS/PAGE. Following transfer, the blocked membrane was incubated with anti-Rad51 antibody (1:1000), which was detected by using the Amersham ECL reagent and peroxidase-conjugated donkey anti-rabbit immunoglobulin (1:2000 dilution).

Immunofluorescence Microscopy of Single Cells. Cells were washed once with PBS and resuspended at 4×10^5 cells per ml, and 0.5-ml aliquots were centrifuged onto glass slides at 800 rpm for 4 min in a Cytospin 3 (Shandon, Pittsburgh). Cells were fixed for 30 min at room temperature in 3.7% paraformaldehyde in PBS, then for 30 min at −20°C in methanol. The slides were incubated for 30 min at room temperature with rabbit anti-Rad51 serum (1:50 dilution into PBS/1% bovine serum albumin); washed three times, 10 min per wash, with PBS; incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (Southern Biotechnology Associates; diluted 1:100 in PBS) for 30 min; washed with PBS; counterstained 5 min with 200 ng/ml 4′,6-diamidino-2-phenylindole (DAPI); washed; and mounted in antifade solution {90% (vol/vol) glycerol/0.1 M Tris·HCl, pH 8.0/2.3% 1,4-diazabicyclo[2.2.2]octane (DABCO)}. In some experiments cells were stained with Texas red-labeled goat anti-mouse IgM or IgG (μ- or γ-chain-specific; Southern Biotechnology) and washed three times with PBS prior to applying rabbit anti-Rad51 antiserum and FITC-labeled secondary antibody. Observation and data analysis were carried out as described by Haaf *et al.* (29). The following control experiments showed that staining was specific: no signal was visualized without inclusion of the primary antibodies; no signal was visualized when primary antibodies preadsorbed with purified recombinant Rad51 were used; and clear specific staining was evident when antibodies affinity purified by binding to purified recombinant Rad5 were used.

***In Situ* Immunofluorescence and Immunohistochemical Staining of Splenic Sections.** Female C57BL/6 mice, 6–12 weeks old, were immunized intraperitoneally with 100 μg of alum-precipitated (4-hydroxy-3-nitrophenyl)acetyl (NP)-chicken gamma globulin together with 2×10^9 heat-killed *Bordetella pertussis* cells (32). Mice were sacrificed on days 2, 3, 5, 7, 9, and 12 after immunization, and spleens were removed, embedded in Tissue-Tek OCT (Baxter Scientific), frozen on dry ice, and stored at −70°C. Cryostat sections (10 μm) were cut and mounted onto ProbeOn Plus slides (Fisher Scientific), fixed in 3% paraformaldehyde for 1 h, and after three rinses (5 min each) in distilled water were dehydrated by sequential rinses (5 min each) in 30%, 75%, and 95% ethanol, air-dried, and stored at −70°C. Sections were rehydrated 10 min in PBS, permeabilized by incubation for 30 min in 0.3% Triton X-100 in PBS, blocked for 1 h at room temperature in 10% dried milk powder in PBS, and incubated with 100 μl of anti-Rad51 antibodies in a humidified environment for 1 h at room temperature or overnight at 4°C. After a 10-min rinse in fresh blocking solution, sections were incubated with 100 μl of goat anti-rabbit IgG antibodies conjugated to Texas red and, in some cases, with FITC-conjugated goat anti-mouse IgG or IgM antibodies; antibodies were diluted 1:100 in blocking solution. After incubation for 1 h at room temperature in a dark, humidified environment, tissue was rinsed for 10 min in PBS then 5 min in distilled water and left to air dry. Sections were mounted in antifade solution to prevent photobleaching. Control studies were conducted as above without inclusion of the primary antibodies, and no specific staining was seen in any of the control sections. To visualize germinal centers, sections were first incubated for 1 h at room temperature in a blocking solution consisting of 10% goat serum (GIBCO) in PBS, then with 100 μl of biotinylated peanut agglutinin (PNA; Pierce; diluted 1:100 in blocking solution) in a humidified environment for either 1 h at room temperature or overnight at 4°C. Sections were rinsed for 10 min in fresh blocking solution and then incubated for 1 h at room temperature in a humidified environment with 100 μl of streptavidin-bound peroxidase [made by preincubating 20 μg/ml streptavidin (Pierce) and 40 μg/ml biotinylated horseradish peroxidase (Pierce) for 30 min at room temperature in blocking solution]. Sections were rinsed 30 min in PBS, then incubated with 1 ml of 3,3′-diaminobenzidine (DAB) substrate solution [0.8 mg/ml DAB (Sigma) and 0.009% H_2O_2 in 100 mM Tris·HCl, pH 7.5]. Color development was usually visualized within 1 h and was stopped by rinsing the sections in distilled water. Sections were air-dried and mounted in Permount (Fisher).

RESULTS

Induction of Rad51 Protein in LPS-Stimulated Murine Primary B Cells. Culture with LPS induces primary murine B cells to switch efficiently from IgM (μ) to IgG (γ) expression, and typically 25–35% of cells switch during 4–5 days of culture. To determine whether induction of switch recombination affected Rad51 expression, we assayed Rad51 protein levels in LPS-activated murine primary B cells by immunoblotting. As shown in Fig. 2, Rad51 protein cannot be detected at day 0, prior to LPS culture; appears at day 1; is greatly enhanced at days 2 and 3; and begins to decline at day 4. Rad51 protein is therefore LPS inducible in murine primary B cells.

Rad5$^+$ Foci Are Induced in LPS-Activated Murine Primary B Cells and Correlate with Size and Morphology of the Nucleus. We used immunofluorescence microscopy to analyze expression of Rad51 at days 0–6 of LPS culture. Data in Table 1 are compiled from analysis of 900-2000 cells at each day of culture, and Fig. 3 shows examples of cells observed. At day 0, prior to cell activation, no Rad51 signal could be detected. By

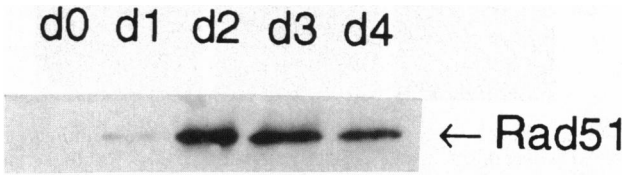

FIG. 2. Rad51 expression in LPS-activated primary murine B cells. An imunoblot of extracts from days 0, 1, 2, 3, and 4 of LPS culture is shown.

Table 1. Kinetic analysis of Rad51$^+$ foci and expression of antibodies of the μ and γ isotypes

Day	% stained					Cells counted
	Rad51$^+$	μ^+	γ^+	Rad51$^+\mu^+$	Rad51$^+\gamma^+$	
0	0	37.0	0.8	0	0	1650
1	1.3	45.9	2.6	0.7	0.1	2005
2	19.3	70.2	6.4	18.1	2.9	1260
3	19.8	58.8	13.7	17.7	5.4	995
4	18.2	23.8	16.8	8.1	2.9	905
5	15.0	22.9	22.7	8.1	2.5	1272
6	14.7	15.8	32.3	5.9	3.0	1140

Cells were assayed at days 0–6 of LPS culture, and data were pooled from three separate experiments. Columns list the percentage of total cells counted that stained with each antibody or pair of antibodies. Double-stained Rad51$^+\mu^+$ and Rad51$^+\gamma^+$ cells were counted separately.

day 1 of LPS culture, Rad51$^+$ foci were evident in the nuclei of a small fraction (1.3%) of cells. At day 2, the fraction of nuclei containing Rad51$^+$ foci increased sharply, to 19.3%, and this number remained constant at days 3 and 4, when many cells were activated and mitotic cells could be easily found. The fraction of Rad51$^+$ cells began to decline at days 5 and 6, when the primary cells began to die. The foci observed are due to Rad51 staining, as no foci could be detected when cells were allowed to react with preimmune serum instead of anti-Rad51 antiserum, or stained with secondary antibody alone (data not shown).

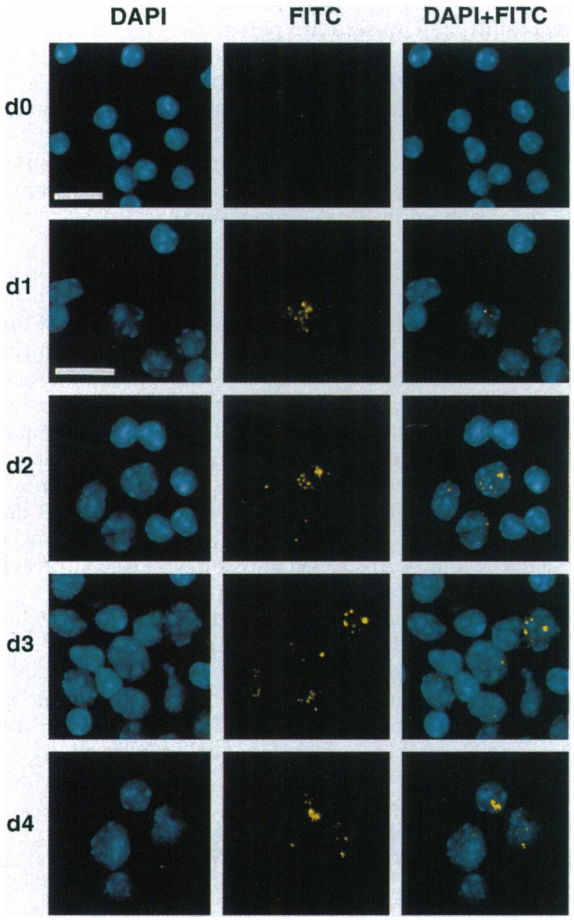

FIG. 3. Rad51$^+$ foci in nuclei. Immunofluorescent staining was assayed at days 0, 1, 2, 3, and 4 of LPS culture. (*Left*) DAPI (blue) stain of nuclear DNA. (*Center*) FITC (yellow) staining by anti-Rad51 antibodies. (*Right*) Merged image of FITC and DAPI staining. [The bars at the bottom of the first row (d0) and second row (d1) represent 20 μm; magnification at d2–d4 was the same as d1].

Throughout the course of LPS culture, Rad51 staining correlated with the size and morphology of the nucleus. At day 0, before activation, all nuclei were small and stained densely with DAPI. Nuclei of B cells that are activated to form blast cells become enlarged and exhibit a diffuse DAPI staining pattern, and Rad51$^+$ foci were restricted to nuclei of this distinctive size and nuclear morphology (see Fig. 3); the numbers of both Rad51$^+$ foci and large blast cells peaked between days 2 and 4. T cells and a fraction of B cells are not activated by LPS; and these cells, which displayed a condensed and intense nuclear DAPI staining pattern throughout the course of culture, were never observed to contain Rad51$^+$ foci. Activated cells of size intermediate between resting B cells and blast cells were evident at day 1 and all subsequent days, but these cells rarely exhibited Rad51$^+$ foci.

Kinetics of Rad51 Expression in μ- and γ-Positive Cells. To explore the relation between Rad51$^+$ foci and switch recombination from IgM (μ) to IgG (γ) expression in LPS-cultured cells, we enumerated cells stained with anti-μ or anti-γ antibodies and anti-Rad51 at each day of culture. As shown in Table 1, prior to LPS culture 37% of cells were μ^+, essentially no cells were γ^+, and no cells were Rad51$^+$. At days 1 and 2, the fraction of μ^+ cells peaked and anti-Rad51 antibodies stained almost exclusively μ^+ cells. At days 3 and 4, the fraction of μ^+ cells declined while the fraction of γ^+ cells increased, indicating that switch recombination had taken place. A graph of the percentage of cells stained with anti-Rad51, anti-μ, or anti-γ antibodies relative to the maximum number of cells staining with that reagent (Fig. 4*A*) showed that there is a surge in the number of cells containing Rad51$^+$ foci between days 1 and 2, followed by a decline in μ^+ cells after day 2. A graph of the percentage of double-stained cells normalized to total cells of that isotype (Rad51$^+\mu^+/\mu^+$ or Rad51$^+\gamma^+/\gamma^+$; Fig. 4*B*) showed two important points: (*i*) even though the fraction of μ^+ cells decreased throughout the course of culture, the ratio of Rad51$^+\mu^+$ cells to all μ^+ cells constantly increased; (*ii*)

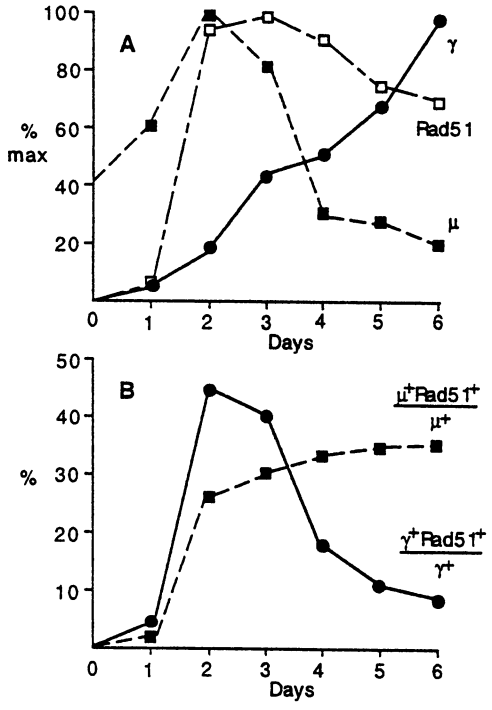

FIG. 4. Kinetics of Rad51, μ, and γ staining of LPS-activated primary B cells. Data were compiled by counting 900-2000 stained cells at each day of LPS culture. (*A*) Percentage of Rad51$^+$ (□), μ^+ (■), and γ^+ (●) cells, relative to maximum number of cells staining positive for each protein. (*B*) Percentage of double-positive cells normalized to total cells of each isotype. ■, μ^+Rad51$^+/\mu^+$; ●, γ^+Rad51$^+/\gamma^+$.

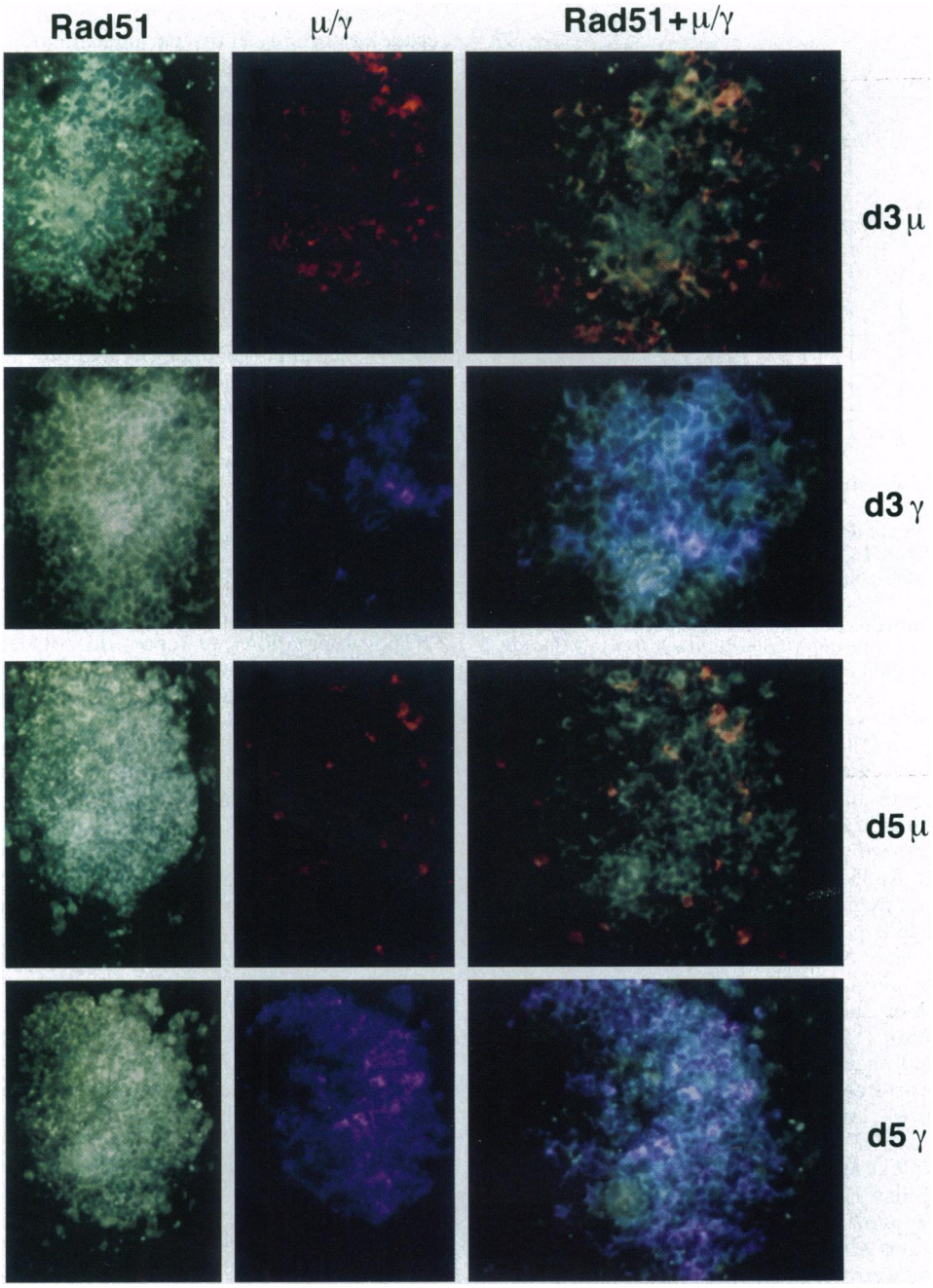

FIG. 5. Localization of Rad51, μ, and γ staining of murine splenic sections. Splenic sections from days 3 and 5 after primary immunization were stained as follows. (*Left*) With anti-Rad51 antibodies alone (green). (*Center*) With anti-μ (orange) or anti-γ (purple) antibodies. (*Right*) Merged images. (The bars at the bottom of each panel represent 50 μm.)

conversely, although the fraction of γ^+ cells increased throughout the course of culture, the ratio of Rad51$^+\gamma^+$ cells to all γ^+ cells constantly decreased. If Rad51$^+$ foci correlated only with cell proliferation and not with active recombination, one would expect an *increase* in the ratio of Rad51$^+\gamma^+$ cells to γ^+ cells during the course of culture, as γ^+ cells continue to proliferate. Although these data do not directly demonstrate a causal relationship between Rad51$^+$ foci and switch recombination, they are consistent with induction of Rad51 in μ^+ cells before switching and down-regulation of Rad51 in γ^+ cells after switching.

Anti-Rad51 Antibodies Stain B Cells Undergoing Isotype Switch Recombination *in Vivo*, but Not B Cells Undergoing Somatic Hypermutation. *In vivo*, B cells undergo class switch recombination and somatic hypermutation in distinct regions of the spleen. In the early days of a primary immune response, antigen-activated B cells associated with the peri-arteriolar lymphoid sheath (PALS) make intimate contact with helper T cells, expand in number, and carry out isotype switch recombination. Subsequently these B cells migrate to lymphoid follicles, where they carry out somatic hypermutation in histologically distinct regions called germinal centers (reviewed in ref. 33).

Using immunofluorescent microscopy, we visualized splenic sections that had been stained with anti-Rad51 antibodies. In sections taken as early as day 2 after immunization, well-defined clusters of cells clearly reacted with this antibody, as shown in Fig. 5). Multiple clusters of staining cells were visible in each splenic section, and from the sizes of the clusters we estimated each to contain 400–600 cells. Kinetic analysis showed that switching occurred between days 3 and 5 after immunization: sections taken at day 3 contained many clusters

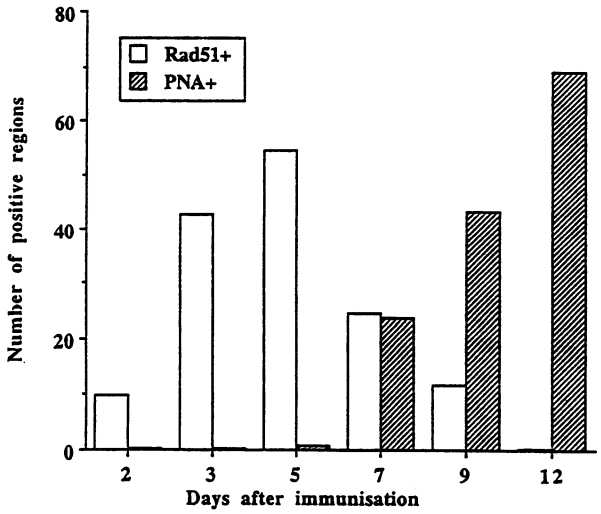

FIG. 6. Kinetic analysis of Rad51 and PNA staining. Bars represent the mean number of Rad51$^+$ regions (open columns) and PNA$^+$ germinal centers (hatched columns) visible; nine or more sections were examined at each time point. Double-staining with anti-Rad51 and anti-λ light chain antibodies correlated the kinetics and localization of Rad51 expression with the λ-dominated response to the NP hapten used in immunization (data not shown).

of μ^+ cells but few clusters of γ^+ cells, whereas sections taken at day 5 contained essentially no μ^+ clusters but an abundance of γ^+ clusters. Simultaneous staining of the sections with anti-Rad51 and anti-μ or anti-γ antibodies showed that at day 3, Rad51 staining colocalized with μ^+ staining; and at day 5, Rad51 staining colocalized with γ^+ staining. Moreover, at day 5 all γ^+ clusters were Rad51$^+$, and *vice versa*. Rad51 staining therefore localized to regions of the spleen where switch recombination occurred.

Germinal centers, sites of somatic hypermutation, can be readily visualized by staining with the lectin peanut agglutinin (PNA). Enumeration of Rad51$^+$ regions and PNA$^+$ germinal centers in splenic sections taken from days 2–12 after primary immunization showed that the kinetics of Rad51 staining correlated with switch recombination, but not with somatic hypermutation (Fig. 6). Like switch recombination, Rad51 staining peaked at days 3–5, while PNA-staining germinal centers were first evident at day 5 and increased in both size and number through day 12, a time course which correlated well with published reports (34, 35). Moreover, incubation of adjacent splenic sections with either anti-Rad51 antibodies or biotinylated PNA showed that Rad51$^+$ regions and PNA-staining germinal centers did not colocalize (data not shown).

DISCUSSION

Anti-Rad51 antibodies stain foci that localize to the nuclei of switching B cells. These foci appear qualitatively similar to the Rad51$^+$ foci induced by DNA damage in mammalian cell lines (29), although the B-cell foci are slightly fewer in number and larger in size. In both cases, the Rad51$^+$ foci appear to correlate with ongoing recombination and/or repair. Similar foci, stained with different specific antibodies, are visualized in the contexts of splicing and transcriptional regulation (e.g., refs. 36–39). It is quite possible that the foci we observe will prove functionally relevant to switch recombination.

Rad51 defines a family of recombination proteins which share functional, sequence, and/or structural similarity, and the polyclonal antibodies that we have used recognize not only Rad51 but also other recombination proteins that share conserved domains. It is therefore possible that Rad51 may not be the only protein visualized by staining with these antibodies. Nonetheless, as we obtain an identical staining pattern with crude serum and affinity-purified antibodies, any other protein visualized must be highly related to Rad51.

Induction of switch recombination in primary B cells results in a dramatic increase in Rad51 expression. Experiments in progress suggest that at least some of the increase in Rad51 gene expression in switching B cells is regulated at the level of transcription. In contrast, in cells treated with agents that damage DNA, Rad51 protein levels remain constant even as foci appear in the nuclei (29).

The kinetics and pattern of Rad51 staining correlate Rad51 with the restricted period of B-cell development when switch recombination occurs. Rad51$^+$ foci were observed in both μ^+ and γ^+ activated primary B cells, but they appeared to be extinguished subsequent to switch recombination. Similarly, in splenic sections, clusters of cells that stained brightly with anti-Rad51 antibodies colocalized early with μ expression and later with γ expression, but disappeared before the peak of germinal center development. The absence of germinal center staining by anti-Rad51 antibodies shows that Rad51 expression is not coincident with somatic hypermutation. Nor does Rad51 staining correlate simply with cell proliferation, as B cells within the germinal center proliferate rapidly, but they are not stained with anti-Rad51 antibodies.

The observations we report do not demonstrate a causal relationship between Rad51 expression and class switch recombination, but they do raise the possibility of such a relationship. If Rad51 does function in switch recombination, what might its role be? One appealing notion is that Rad51 molecules might converge at regions of DNA where recombination is about to occur. We suspect that this may not be the case, because when Rad51 foci are induced in mammalian cells in response to DNA damage, the foci do not colocalize with sites of repair synthesis (29). In double-strand break repair in yeast, Rad51 may function as part of a recombination complex that includes Rad52, Rad55, and Rad57 (40–42). The ubiquitin-conjugating enzyme, Ubc9, has been shown to interact with Rad51 and to localize to synaptonemal complexes in meiotic murine spermatocytes (43); mammalian Rad52 has also been suggested to interact with Rad51 (25). Identification of proteins that specifically interact or colocalize with Rad51 in switching B cells may reveal some of the other participants in switch recombination.

This research was sponsored by National Institutes of Health Grants GM39799 (N.M.), CA39238 (C.M.R.), and 5R01HG00272 (D.C.W.).

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