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Research Article

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***Staphylococcus aureus* Cowan I**

Potent Stimulus of Immunoglobulin M Rheumatoid Factor Production

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Abstract

These studies demonstrate that *Staphylococcus aureus* Cowan I (SAC), a protein A-positive Staphylococcal strain, is a potent and consistent inducer of IgM rheumatoid factor production by normal human peripheral blood mononuclear cells. The frequency and magnitude of this response greatly exceeded that of parallel cultures stimulated with pokeweed mitogen or the protein A-negative *S. aureus* Wood strain, although all three agents induced a similar amount of total IgM. Cell fractionation studies indicated that SAC-induced IgM rheumatoid factor is T cell-dependent. The striking ability of SAC to induce IgM rheumatoid factor may relate to its protein A content, since cultures stimulated with protein A-coupled sepharose beads also consistently produced this autoantibody. Thus SAC is a new probe of in vitro IgM rheumatoid factor production and its use has provided further evidence that most healthy individuals harbor precursors of IgM rheumatoid factor secreting cells. Unlike other polyclonal activators, SAC is unique in its capacity to bind immunoglobulin, a property that may account for its prominent anti-IgG inducing capacity.

Introduction

Rheumatoid factors (RF)¹ are antiglobulins directed against the Fc fragment of autologous IgG. Attention was originally focused on these autoantibodies because of their possible pathogenic role in rheumatoid arthritis, where IgM RF were first discovered (1, 2). Evidence supporting this view was provided by the findings that RF (a) was produced locally by synovial tissue (3), (b) could be found in immune complexes in synovial fluid (4), and (c) when complexed with aggregated IgG could activate the classical complement cascade (5).

However, RF are not infrequently found in the serum of nonarthritic clinical disorders, particularly those associated with chronic inflammation and certain bacterial infections (reviewed in reference 6). This indicated that the production of this au-

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1. Abbreviations used in this paper: EBV, Epstein-Barr virus; PBMC, peripheral blood mononuclear cells; PWM, pokeweed mitogen; RF, rheumatoid factor; RT, room temperature; SAC, *Staphylococcus aureus* Cowan I; SAW, *S. aureus* Wood strain.

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toantibody was not a priori associated with the development of rheumatoid arthritis, and furthermore suggested that RF production might be related to processes, as yet poorly elucidated, which occur during inflammation. The traditional concept, promulgated over the years, has been that RF is produced secondary to an altered structural configuration of the IgG molecule as might occur after its complexing with complementary antigen (7).

One alternative explanation is that RF, and perhaps other autoantibodies, may arise as a consequence of polyclonal B cell activation. It has been observed that several well-characterized polyclonal B cell activators like pokeweed mitogen (PWM) (8), Epstein-Barr virus (EBV) (9, 10), lipopolysaccharide (10), and bacterial peptidoglycan (11, 12) elicit the production of RF by murine or human lymphocytes. Such studies demonstrated that lymphocytes of some healthy donors may make RF in response to polyclonal B cell activators. Thus, they suggest that self-reactive clones are present in the normal host and, more particularly, that the precursors of RF-secreting cells might be normal constituents of the B cell repertoire.

Formaldehyde-fixed *Staphylococcus aureus* Cowan I (SAC), a staphylococcal strain rich in protein A, is a polyclonal B cell activator that has provided considerable insight into the sequence of events in and factors requisite for normal B cell activation (reviewed in reference 13). During the course of in vitro studies aimed at characterizing the properties of antibodies elicited by this agent, we observed that SAC elicited IgM-RF production by peripheral blood mononuclear cells (PBMC) of all but one healthy donor surveyed. Our results underscore an important role for protein A in this response and provide further compelling evidence that precursors of IgM-RF secreting cells are constituents of the normal B cell repertoire. Additionally, the data suggest new mechanisms by which such cells are activated in vivo.

Methods

Blood donors. 15 healthy adult volunteers (age range 18-52 yr) served as lymphocyte donors. There was no history of arthritis, other rheumatic disease, recent infectious illness, or drug use.

Polyclonal activators. PWM was purchased from Gibco (Grand Island, NY). Formalin-treated SAC and *S. aureus* Wood strain (SAW) were purchased from Calbiochem-Behring Corp. (LaJolla, CA). SAC and SAW were stored as stock solutions in phosphate-buffered saline (PBS) and were washed several times before their addition to cultures. Soluble protein A (Pharmacia Fine Chemicals, Piscataway, NJ) was conjugated to cyanogen-activated Sepharose 4B (Pharmacia Fine Chemicals) by standard technique (14). The protein A-conjugated beads were stored at 4°C and likewise were washed several times before use in culture.

Preparation of cells. PBMC were obtained from the blood of healthy volunteers by Ficoll-Hypaque gradient separation. In certain experiments, PBMC were separated into T cell- and B cell-enriched fractions by rosetting with neuraminidase-treated sheep erythrocytes followed by centrifugation on a Ficoll-Hypaque gradient as previously described (15). The B cell fraction was then incubated with a cocktail of OKT3, OKT11

(Ortho Pharmaceutical Corp., Raritan, NJ), and Leu M3 monoclonal antibodies (Becton-Dickinson & Co., Sunnyvale, CA). They were washed extensively and then applied to a 7-ml column of Sepharose 6MB (Pharmacia Fine Chemicals) coupled with goat anti-mouse IgG to rigorously deplete T cells and reduce the number of contaminating monocytes. The nonadsorbed cell fraction, hereafter called B cells, contained <1% OKT3- or OKT11-positive cells and <3% Leu M3-positive cells (monocytes) as determined by flow cytometry (16).

Cell cultures. Cells were prepared in RPMI 1640 medium (Microbiological Assoc., Walkersville, MD) supplemented with 10% fetal calf serum (KC Biological, Inc., Lenexa, KA), penicillin-streptomycin, 1% vol/vol, and glutamine, 2% vol/vol. In some experiments, cycloheximide (25 μ g/ml) was incorporated into the medium. Replicate cultures of 2×10^5 PBMC in 0.2 ml medium were established in 96-well round-bottom tissue culture plates (Linbro; Flow Laboratories, McLean, VA) in the presence of varying concentrations of the polyclonal activators or additional complete medium. Cultures were maintained at 37°C in a humidified atmosphere containing 5% CO₂ and 95% air. After 7 to 8 d, cultures were centrifuged and replicate supernatants were pooled and stored at -20°C until analyzed for total IgM, IgG, and IgM RF. In experiments designed to examine the T cell dependency of SAC-induced IgM-RF production, T cells alone, B cells alone, or admixtures of T cells and B cells were cultured as described above.

Assay for supernatant IgM and IgG. Supernatant IgM and IgG were determined by an enzyme-linked immunosorbent assay (ELISA). To wells of a 96-well polystyrene microtiter plate designed for ELISA (Costar, Cambridge, MA) were added 0.1 ml of a 10- μ g/ml solution of affinity-purified goat anti-human IgG or anti-IgM (Tago, Burlingame, CA) in pH 9.4 carbonate buffer. After incubation overnight at 4°C, the wells were drained and "blocked" with 0.1 ml of 1% bovine serum albumin (BSA)/PBS solution for 1 h. Then dilutions of (a) a purified IgG or IgM standard (Tago), (b) neat, 1:10, and 1:100 dilutions of unknown supernatants, or (c) medium alone were added to replicate wells. All dilutions were made in complete culture medium. After 3 h at room temperature (RT), wells were drained and washed four times with 0.05% Tween/PBS. This was followed by the addition of 0.1 ml peroxidase-conjugated goat anti-human Ig (Sigma Chemical Co., St. Louis, MO) diluted 1:1,000 and further incubation overnight at 4°C. After three washes the next day, 0.4% *O*-phenylenediamine and 0.012% H₂O₂ were added and the plates incubated in the dark for 10 min. Color development was read at 450 nm on a multichannel spectrophotometer (Titertek; Flow Laboratories). A standard curve was constructed by plotting log optical density vs. log concentration of input Ig. A linear regression line was developed and values for unknowns determined from the standard curve with a computer program.

Determination of IgM-RF. Levels of IgM-RF in culture supernatants were also quantitated by ELISA. Duplicate wells of a polystyrene plate (Dynatech Laboratories Inc., Alexandria, VA) were coated with 10 μ g/ml Fc fragment of human IgG (Cappel Laboratories, Cochranville, PA) in pH 9.6 carbonate buffer. Duplicate control wells received carbonate buffer alone. After incubation for 18-72 h at 4°C, wells were coated with 1% BSA (Sigma Chemical Co.) in PBS. After 3-6 h at RT, a monoclonal IgM-RF standard (kindly provided by Dr. R. Heimer, Jefferson Medical College, Philadelphia, PA) diluted to 100, 50, 25, 10, 5, 2.5, or 1.0 ng/ml in complete medium was added to duplicate Fc-coated and uncoated wells. The monoclonal IgM-RF was obtained from the serum of a patient with Waldenstrom's macroglobulinemia and its preparation previously reported (17). PBMC culture supernatants were added in parallel to duplicate Fc-coated and -uncoated wells. After 18 h at RT, wells were washed and incubated with 250 ng/ml of affinity-purified goat anti-human IgM (Kirkegaard and Perry Laboratories, Gaithersberg, MD) in BSA-PBS for 2 h at RT. Wells were then incubated for 2 h at RT with 250 ng/ml of biotinylated affinity-purified rabbit anti-goat IgG antibody (Vector Laboratories, Inc., Schiller Park, IL) in Tween/PBS. Avidin-biotinylated horseradish peroxidase complex (Vector Laboratories) was diluted 1:80 in Tween/PBS and added to all wells for a 1-h RT incubation. This was followed by the addition of enzyme substrate indicator, which

consisted of 0.01% hydrogen peroxide with *O*-phenylenediamine (Eastman Kodak Co., Rochester, NY) to each well for 30 min at RT. Color absorbance for each well was read at 450 nm on the multichannel spectrophotometer (Titertek; Flow Laboratories).

The absorbance for each dilution of monoclonal RF was corrected for nonspecific binding by subtraction of the absorbance in corresponding uncoated wells. A standard curve was constructed by plotting log optical density vs. log concentration of input monoclonal RF. The absorbance from wells containing unknowns was similarly corrected and the concentration of RF determined from the standard curve. Values of RF were linear over a range of 2.5-100 ng/ml, with 2.5 ng/ml representing the lower threshold of detection.

Results

In preliminary experiments we determined that optimal IgM-RF secretion occurred on days 7-9 of culture. Indeed, little if any RF was produced prior to day 4. A similar kinetic pattern was observed for total IgM and IgG responses. Additional experiments were carried out to examine the optimal dose of SAC that stimulated RF production. A narrow optimal dose range was consistently observed (range 1:500-1:1,000 vol/vol of stock solution) and this range was similar to that which optimally stimulated total IgM responses.

Frequency and magnitude of SAC-induced IgM-RF production. Next PBMC were cultured in the presence of varying concentrations of SAC, SAW, PWM, or additional medium and IgM-RF responses determined. Data points in Fig. 1 represent peak IgM-RF determinations for each culture additive. SAC induced IgM-RF production (≥ 2.5 ng/ml) in PBMC cultures of 14/15 subjects. This frequency of response was significantly higher than that observed for SAW (3/15, $P < 0.001$, chi-square analysis) and PWM (7/15, $P < 0.01$). Additionally, the magnitude of IgM-RF responses (13.7 ± 2.6 , mean \pm SEM) in SAC-stimulated cultures was greater than that of SAW- (0.9 ± 0.2 ng/ml, $P < 0.001$, Student's *t* test) or PWM (2.2 ± 0.4 ng/ml, $P < 0.01$)-stimulated cultures.

Detection of IgM-RF does not reflect carryover of soluble protein A to ELISA. Protein A has been reported to elute off SAC during the course of 3-d cultures (18). If soluble protein A was carried over in the supernatants before the determinations of RF, falsely elevated values of RF could have been observed. Soluble protein A could have bound to the Fc-coated wells and subsequently bound the rabbit or goat IgG-developing antibodies. That this was not the case was already suggested by the kinetic studies in which little to no RF production was observed before day 4. Had soluble protein A escaped into the supernatants, this would likely have occurred by this time. Nevertheless, a series of experiments was carried out to exclude this possible technical pitfall. First, IgM-RF production was not detected in supernatants of SAC-stimulated PBMC cultured in the presence of cycloheximide (Fig. 2). Secondly, SAC was incubated for a period of 7 d in medium without PBMC. The medium was recovered in the same fashion as if PBMC were present and was found to contain no RF (Fig. 2). Taken together, these experiments indicated that SAC-induced IgM-RF production was dependent upon active protein synthesis and did not reflect carryover of soluble protein A into the ELISA.

T cell dependency of SAC-induced IgM-RF production. To determine whether SAC-induced IgM-RF was T cell-dependent, cultures of B cells alone, T cells alone (10^4), and an admixture of T cells (10^4) plus B cells (5×10^4) were established in the

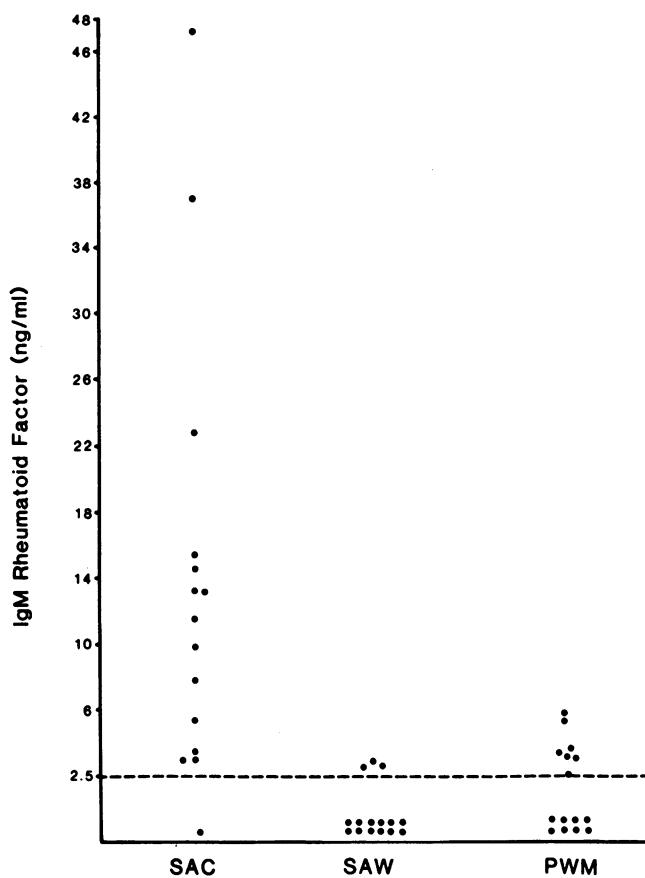


Figure 1. IgM-RF production induced by SAC, SAW, and PWM. PBMC from 15 healthy volunteers were incubated for 7–8 d with varying concentrations of the designated culture additives and supernatants analyzed for IgM-RF by ELISA. Values represent peak responses to each additive. IgM-RF was never detected when cells were incubated in medium alone. The hatched bar signifies the lower threshold of IgM-RF detection (2.5 ng/ml).

presence of SAC and secreted IgM and IgM-RF were determined after 7 d. Results from two representative experiments are shown in Table I. Little IgM-RF was produced when B cells were stimulated with SAC. Although T cell contamination of the B cells was reduced to <1%, it is likely that the small amount of IgM-RF observed reflected helper activity of a few residual T cells, since the same SAC-stimulated B cells also demonstrated a minimal but discernible total IgM response. Nevertheless, addition of T cells, which themselves made no IgM-RF, to B cells resulted in an augmented SAC-induced IgM-RF response. As expected, a parallel increase in total IgM was also observed.

Relationship of SAC-induced IgM-RF response to total IgM responses. The greater capacity of SAC than SAW or PWM to stimulate IgM-RF production did not reflect a greater capacity to induce total IgM responses (Fig. 3). The distribution of total IgM responses induced by SAC was similar to that of SAW ($P > 0.1$, Mann-Whitney test) and to that of PWM ($P > 0.1$). Furthermore, there was no correlation between SAC-induced total IgM and SAC-induced IgM-RF responses observed in individual subjects ($r = 0.03$, $P > 0.1$, data not shown).

IgM-RF by protein A-stimulated PBMC. One interpretation of the vast superiority of SAC over SAW as an inducer of IgM-RF was that protein A was the responsible trigger. Therefore,

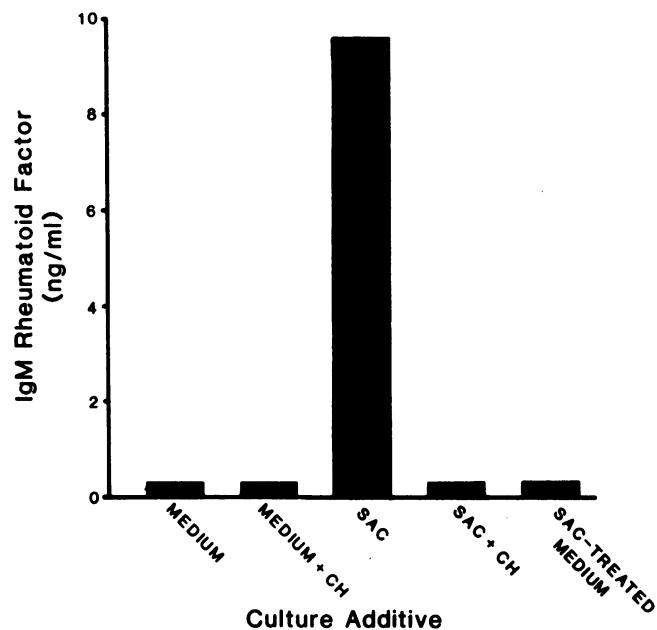


Figure 2. Analysis of SAC-induced IgM-RF production. PBMC were incubated with SAC or additional medium in the presence or absence of cycloheximide (CH), and IgM-RF was determined. Additionally, SAC was incubated in culture medium without PBMC (SAC-treated medium) and the "supernatant" analyzed for IgM-RF.

we sought to determine if protein A was capable of eliciting rheumatoid production. To address this question, we employed protein A-conjugated Sepharose 4B. In this manner, we were able to remove protein A from culture supernatants by centrifugation, and thus avoid the potential problem of carryover of soluble protein A into the ELISA. Protein A Sepharose elicited in vitro RF production in 5/5 experiments of which the data in Fig. 4 are representative. As was true for SAC, a narrow dose-response range was observed and the response was abrogated by

Table I. T Cell Dependency of SAC-induced IgM-RF Production

Cells*	IgM-RF		IgM	
	Culture additive		Culture additive	
	Medium	SAC	Medium	SAC
Exp. 1				
B*	ND‡	4	25	213
T	ND	ND	ND	ND
T + B	ND	14	23	673
Exp. 2				
B	ND	4	19	188
T	ND	ND	ND	ND
T + B	ND	29	29	801

*B, 5×10^4 B cells; T, 10^4 T cells; T + B, 10^4 T cells + 5×10^4 B cells. Cells were cultured for 8 d with SAC or additional medium under conditions described in the text. Supernatants from replicate cultures were pooled and analyzed for IgM-RF and total IgM.

‡ND, none detected.

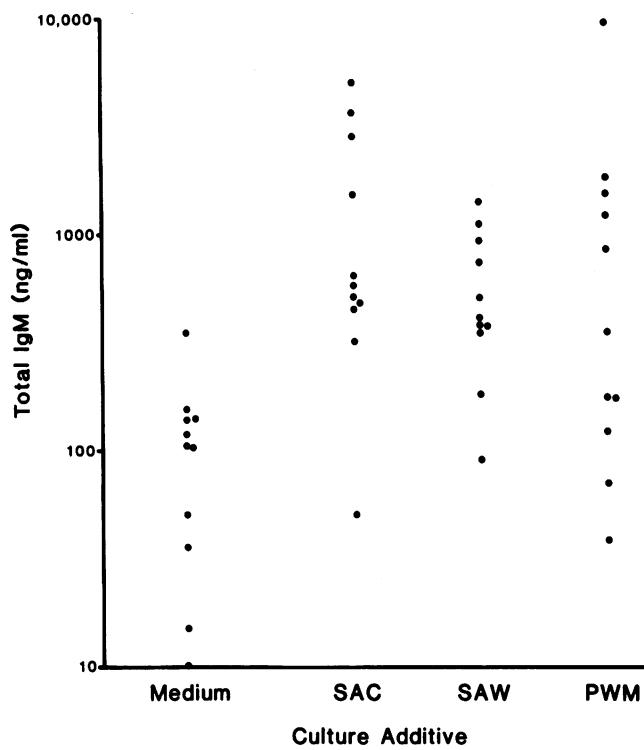


Figure 3. Comparative analysis of total IgM production induced by SAC, SAW, and PWM. PBMC were incubated as described in Fig. 1 and total IgM values were determined by ELISA.

cycloheximide. Unconjugated sepharose beads did not elicit a response.

Discussion

SAC has been extensively investigated as a probe to delineate mechanisms of B cell activation, proliferation, and differentiation (reviewed in reference 13). Its utility relates to its apparent mim-

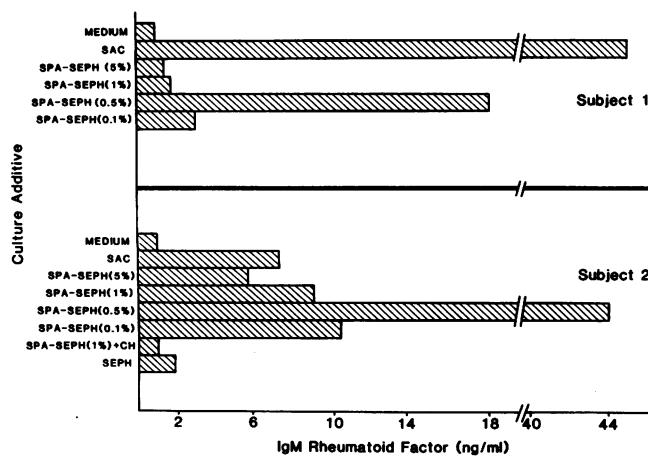


Figure 4. Soluble protein A coupled to sepharose beads induces IgM-RF production. PBMC were incubated with the designated culture additives and IgM-RF was determined. SPA-SEPH, sepharose beads conjugated with soluble protein A; CH, cycloheximide; SEPH, unconjugated sepharose beads.

icry of antigen in the activation of B cells. Thus, the principal mode of activation by SAC involves perturbation of membrane Ig receptors via its protein A moiety (19, 20). SAC is a T cell-independent B cell mitogen but requires T cells or T cell-derived factors to stimulate B cells to undergo polyclonal B cell differentiation (13). Despite its widespread use as a polyclonal B cell activator, there are no reports of its ability to induce RF production.

The current studies are highlighted by the finding that SAC elicited IgM-RF production by PBMC of most healthy human volunteers. SAC-induced IgM-RF production was T cell-dependent, since addition of T cells to B cell cultures markedly augmented this IgM-RF response. Although other polyclonal B cell activators have been reported to induce RF in short-term cultures of normal PBMC, they appear to do so less frequently. PWM elicits IgM-RF by cells from roughly 50% of normal donors (8), a frequency confirmed in the present series. Staphylococcal peptidoglycan, a relatively T cell-independent polyclonal B cell activator, does so in only 25% normal donors (11). The T cell-independent polyclonal B cell activator, EBV, induces RF by lymphocytes from unselected healthy donors in more extended cultures, although the exact incidence is difficult to determine from the published studies (9, 21).

In light of the striking frequency of SAC-induced IgM-RF responses observed, efforts were taken to exclude trivial explanations for our findings. Most importantly, positive RF determinations did not reflect carryover of soluble protein A from supernatant to ELISA. The latter is a possibility suggested by Schuurman et al. (18), who found that small amounts of protein A could be eluted into medium by some lots of SAC during short-term culture. Had this occurred in our cultures, protein A could have bound to the Fc-coated wells and subsequently bound either the rabbit or goat "developing" antibodies. This in turn would have resulted in falsely elevated RF determinations. This possibility was excluded by our observation that (a) SAC-induced RF production was not observed unless PBMC had been cultured for at least 4 d, (b) no RF was ever detected in medium incubated with SAC (but without cells), and (c) cycloheximide consistently abrogated SAC-induced RF production. The latter control additionally indicated that SAC-induced RF production requires active protein synthesis.

In addition to PWM, a model T cell-dependent polyclonal B cell activator, SAC was compared with SAW in its capacity to induce in vitro RF production. Since SAW lacks protein A, we hoped to get an early clue about the importance of protein A in the RF inducing activity of SAC. Our data confirm earlier studies that protein A-negative staphylococcal organisms induce polyclonal B cell activation of human lymphocytes (22). More importantly, our results indicate that RF production is an infrequent concomitant of this type of polyclonal B cell activation, thereby suggesting an important role for protein A in the elicitation of RF production. Our studies showing that protein A uniformly induced RF production (Fig. 4) are consistent with this hypothesis. In these studies, protein A was conjugated to Sepharose beads to circumvent the potential confusion introduced into the ELISA by soluble protein A. Whether or not protein A must be presented in an insoluble form remains to be determined.

The superiority of SAC over PWM or SAW to induce IgM-RF was not due to it being a more potent stimulus of IgM production. All three stimuli elicited comparable amounts of total

IgM production. This finding suggests that qualitative differences in the B cell subsets activated by these three agents likely accounts for the differences in IgM-RF production observed. There is ample evidence that different polyclonal B cell activators may activate distinct subsets of B cells (23). This point is clearly relevant to in vitro RF production where studies have shown that EBV and PWM activate distinct subsets of RF secreting cell precursors (24, 25).

Although the data suggest that protein A is responsible for SAC-induced IgM-RF production, the mechanisms of induction are presently not known. The finding that SAC-induced IgM-RF production did not reflect total IgM production suggests that SAC-induced IgM-RF is not merely a by-product of polyclonal B cell activation. This raises the possibility that SAC elicits IgM-RF production and polyclonal B cell differentiation by separate mechanisms. SAC-induced polyclonal B cell activation appears to be caused by the interaction of protein A with the F(ab')₂ fragment of B cell surface membrane immunoglobulin (19, 20). This type of interaction is distinguished from the more conventional type of binding that has been described between protein A and the Fc fragment of IgG (26, 27, and reviewed in reference 28). Indeed, it now appears that the segments of protein A responsible for these two respective types of Ig binding reside on different parts of the protein A molecule (20). One possibility is that SAC, via the Fc (IgG) binding site on protein A, reacts with IgG in the fetal calf serum supplemented medium or IgG secreted by the responding lymphocytes. Such an interaction could effectively alter the configuration of the Fc fragment of IgG and render it immunogenic, a possibility suggested by earlier studies that examined the immunogenic properties of SAC in the rabbit (29). According to this hypothesis, SAC- or protein A-induced IgM-RF production would be caused by an antigen-specific mechanism (altered IgG) rather than by polyclonal B cell activation after an interaction between the F(ab')₂ binding site on protein A and lymphocyte surface immunoglobulin. This would represent a novel situation wherein protein A and an antibody it elicits (RF) display a similar binding specificity, i.e. for the Fc fragment of IgG. In this regard, RF and protein A show preferential binding with IgG₁, IgG₂, and IgG₄ (6, 28) subclasses and also appear to bind to the same site in the CH2 and CH3 domain of the IgG molecule (30).

Irrespective of the mechanism that is ultimately uncovered, our data indicate that most healthy individuals harbor precursors of RF secreting cells. The in vivo interaction of such cells with protein A might also lead to RF production. Since several microbial organisms contain protein A or protein A-like molecules (28), our results could explain the frequent appearance of RF during the course of certain bacterial infections.

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