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New ELISA for B Cell–Activating Factor

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BACKGROUND: The B cell–activating factor of the TNF family (BAFF) is upregulated in autoimmune diseases, but a number of conflicting results have cast doubts on the reliability of the ELISA protocols currently used for its quantification. This situation led us to develop a new ELISA for the measurement of BAFF.

METHODS: BAFF was purified for use alongside nonglycosylated recombinant BAFF. Two monoclonal antibodies (mAbs) and two polyclonal antibodies (pAbs) to BAFF were used.

RESULTS: The optimization process showed that the pAb format was preferable to the mAb format as capture antibody, because the pAbs recognized the glycosylated as well as the nonglycosylated forms of BAFF. The most efficient pair of Abs involved using the unconjugated form of a goat pAb to capture BAFF and the same biotinylated goat pAb to detect bound BAFF. This ELISA was not influenced by the presence of rheumatoid factor.

CONCLUSIONS: This new ELISA helped provide insights into why serum concentrations of BAFF vary between studies for a given population of patients. It is a reliable tool for the management of the diseases in which BAFF is an indication of response to therapy.

Substantial progress in the understanding of systemic lupus erythematosus (SLE),[3] rheumatoid arthritis (RA), and primary Sjögren syndrome (SS) occurred with the discovery of 2 new tumor necrosis factor family members. The first of these is referred to as BAFF, for B cell–activating factor of the tumor-necrosis factor family *(1)*, and the second as APRIL, for a proliferation-inducing ligand *(2)*.

Soluble variants of BAFF are the major contributors of its activity, through regulating the survival of B cells *(3)*. The importance of BAFF to B cells was confirmed when mice transfected with the gene *BAFF* manifested excessive lymphocytic proliferation, together with autoantibody production *(4)*. Further to these observations, excessive quantities of BAFF were found in sera and cerebrospinal fluids of SLE patients *(5)*, sera and synovial fluids (SFs) of RA patients *(6)*, and sera and salivary glands of SS patients *(7)*. Additional evidence that BAFF is involved in autoantibody production includes its upregulation by B-cell depletion in patients with RA *(8)*, and the inverse correlation between serum concentrations of BAFF before depletion of B cells and the length of time before their return in the blood of patients with SS *(9)*.

There exists the issue of why the serum concentrations of BAFF remain within, or below, normal range in a proportion of SLE patients *(9)*. Also intriguing is that increased serum concentrations of BAFF have been associated with the production of rheumatoid factor (RF) by some *(10)* but not other *(11)* authors, or with that of anti-DNA antibody by some *(12)* but not other *(13)* authors.

In addition, estimates of BAFF fluctuate with changes in inflammatory activity, extent of inflammation, and disease classification criteria chosen by the investigators. Pathological features may also influence serum concentrations of BAFF. Thus, receptor occupancy on B cells by BAFF *(14)*, its urinary excretion in case of renal failure *(13)*, and sequestration within immune complexes *(15)* all hinder assessment of BAFF. More importantly, flaws or variations in an ELISA might explain variations in the assessment of BAFF. For example, the absorbance is affected by serum concentration of Ig, affinity of the monoclonal Ab (mAb) selected for capturing BAFF, specificity of the polyclonal Ab (pAb) used for its detection, and the quality

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[3] Nonstandard abbreviations: SLE, systemic lupus erythematosus; RA, rheumatoid arthritis; SS, Sjögren syndrome; BAFF, B cell–activating factor; APRIL, proliferation-inducing ligand; SF, synovial fluid; RF, rheumatoid factor; mAb, monoclonal antibody; pAb, polyclonal antibody; rBAFF, recombinant BAFF; biot, biotinylated; aa, amino acid; pBAFF, purified BAFF; PVDF, polyvinylidene difluoride; HRP, horseradish peroxidase; ECL, enhanced chemiluminescence; CB, Cibacron blue; WB, Western blotting; PBST, PBS containing 0.1% Tween 20; TFMS, trifluoromethanesulfanic acid.

of the calibrators used for constructing the curves. Another potential for variations is that, due to their 50% homology *(1, 2)*, BAFF and APRIL may compete for binding to anti-BAFF Ab, leading to falsely lower concentrations of BAFF. Adding to the complexity is that posttranslational modifications of BAFF may alter its recognition in the ELISA by the Abs. Indeed, evidence exists that BAFF takes on unpredictable glycoforms *(16)*, alternate Δ3 *(17)*, intergenic splice variants *(18)*, isoforms presently undefined, and combinations of BAFF and APRIL *(19)*.

In practice, the Abs used in an individual immunoassay kit may bind to 1 variant of BAFF, but not to another, while those used in a second kit may recognize this latter variant, but not the former. We set out to address deficiencies in the current ELISA protocols and fixed the thresholds for positivity using 2 different kits at 2 SDs above the mean of 100 normal sera. The values above 2 SDs were scored "positive," and those below, "negative." A large group of sera enabled us to compare the 2 kits. There were as many +/− as −/+ mismatches between the 2 series of results, casting doubts on the suitability of the ELISA protocols for measurement of BAFF. This prompted us to develop our own ELISA for BAFF quantification. Given the promise held out for treatment of SLE, RA, and SS with BAFF antagonists, we made every effort to identify differences in the fluid-phase forms of this cytokine and to describe the variants of BAFF characteristic of each autoimmune disorder.

Materials and Methods

ANTIBODIES

Based on their large use in the commercial kits, 2 anti-BAFF mAbs were chosen based on their selection by most of the manufacturers. They were designated M64 (Peprotech) and 137314 (R&D Systems). There were also 2 anti-BAFF pAbs, designated rabbit (Upstate) or biotinylated rabbit (biot-rabbit) (ProSci) and goat or biot-goat (both from Antigenix). Combining any of these 4 Abs as the capture agent (first member of the pair) with any of them as the detection agent (second member) provided 16 different combinations. These mAbs and pAbs were representative of commercially available Abs, since most of them had been generated using the same recombinant BAFF (rBAFF) or similar synthetic peptides.

PURIFICATION OF HUMAN BAFF

The full-length molecule of BAFF is a 285-amino acid (285-aa) peptide with 2 potential *N*-glycosylation sites at residues 124 and 242. Cleavage downstream from aa 124 releases a first form of nonglycosylated BAFF, and possibly a second form glycosylated at aa 242. The so-called ΔBAFF lacks several amino acids, and thereby unmasks a third glycosylation site, since threonine comes to be the second aa downstream from asparagine *(20)*. Glycoforms of BAFF may hence be generated by alternate splicing.

Most of the commercially available rBAFF is not glycosylated, necessitating that we purify glycosylated BAFF. U937 cells were taken as the source of BAFF, on the basis that myeloid cells release BAFF into culture supernatants *(21)*. We isolated purified BAFF (pBAFF) from U937-conditioned medium by affinity-purification through a M64 column, as described *(22)*. We verified the purity of rBAFF and pBAFF by SDS-PAGE, where Sypro™ Ruby (Molecular Probes) stained a single 17-kDa band for rBAFF and a single 28-kDa band for pBAFF.

Each of the 2 preparations was electroeluted onto 4 strips of polyvinylidene difluoride (PVDF) membrane (Bio-Rad). After saturation with 5% casein in PBS, each of the 4 anti-BAFF Abs was used to probe the 4 rBAFF and 4 pBAFF strips. The Ab binding was revealed with horseradish peroxidase (HRP)-conjugated donkey antimouse Ig, antirabbit Ig, or antigoat Ig (all from Jackson) and visualized by enhanced chemiluminescence (ECL).

WESTERN BLOTTING

Before SDS-PAGE, proteins were cleared from sera with dye-modified affinity Cibacron blue™ (CB) resin F3G-A *(23)*, according to Bio-Rad instructions. The sera were then dialyzed overnight against PBS and incubated for 60 min with 5 vol of CB-coated agarose beads. One aliquot of supernatant was subjected to SDS-PAGE and another kept for ELISA to ensure that BAFF had not been adsorbed onto the beads.

In Western blotting (WB), 1 vol of CB-treated samples was mixed with 1 vol of 30 mmol/L Tris-HCl buffer, containing 4% SDS and 1% mercaptoethanol, boiled for 5 min, and loaded onto the SDS-PAGE. After washes with PBS containing 0.1% Tween 20 (PBST), the strips were probed for 16 h at 4 °C with the indicated Ab and visualized by ECL using HRP-conjugated Abs toward mouse, rabbit, or goat Ig.

ANALYSIS OF GLYCOSYLATION

The antigen used to generate anti-BAFF pAb in rabbit was a 13-aa peptide, whereas those used to raise the murine M64 and 137314 mAbs and the goat anti-BAFF pAb were *Escherichia coli*–derived BAFF recombinants. We first verified that these 4 Abs recognized glycosylated as well as nonglycosylated BAFF. BAFF purified from culture supernatant was the control for glycosylated BAFF, and rBAFF the control for its nonglycosylated form.

To further determine specificity of the Abs, pBAFF was deglycosylated. We used anhydrous trifluoromethanesulfanic acid (TFMS) to cleave the glycans, with RNase B as a positive control *(24)*. In a second method, we used the enzyme *N*-glycosidase F (Boehringer) with serum IgA as an affinity-purified positive control, as described *(25)*.

CROSS-REACTIVITY BETWEEN BAFF AND APRIL

Structural similarities between BAFF and APRIL are greater than would be indicated by sequence homology; this fact prompted us to test the 4 anti-BAFF Abs with rAPRIL (R&D Systems), using an anti-APRIL mAb (Peprotech) as a positive control in the WB and ELISA. We resolved 2 μg rAPRIL by 10% SDS-PAGE and transferred it to a PVDF membrane. Unbound sites were blocked with 3% nonfat dry milk in PBST, and the strips were incubated overnight at 4 °C with the 4 anti-BAFF Ab and anti-APRIL Ab (Peproteck) at 0.1 mg/L. Binding was detected with the corresponding secondary Ab, revealed by HRP-labeled streptavidin, and quantitated by ECL. In the ELISA, we coated microplates overnight at 4 °C with 0.5 mg/L of the captive goat anti-BAFF pAb in carbonate bicarbonate buffer. The wells were saturated with BSA for 3 h, and concentrations of 0.05–50 μg/L of rAPRIL were added. After 2-h incubation at 37 °C, we dispensed 0.1 μg/L detection biot-goat into the wells and left the plates at 37 °C for 90 min.

SELECTION OF THE OPTIMAL PAIR OF ANTIBODIES

It was not possible to distinguish between the capture mAb and the detection mAb with the HRP-conjugated antimouse Ab, if the 2 mAbs were paired. Consequently, the 137314 + M64 and M64 + 137314 combinations were discounted from the 16 theoretical combinations. The same principle was applied with regard to the M64/M64, 137314 + 137314, goat + goat, and rabbit + rabbit pairings. In addition, the homology between rabbit and goat IgG could induce cross-reactivity of the second-layer antirabbit IgG Ab with goat IgG and of the second-layer antigoat IgG Ab with rabbit IgG. The rabbit + goat and goat + rabbit pairs were thus believed unsuitable for this assay and provisionally excluded.

These considerations left 8 pairs for the experiments. The view prevails, however, that mAbs are more efficient than pAbs in capturing proteins. For this reason, we initially chose M64 and 137314 for the assessment. Because the quality of Ab coating the plate is critical, however, we kept the following combinations open for consideration: rabbit + 137314, rabbit + M64, goat + 137314, goat + M64, rabbit + biot-goat, goat + biot-rabbit, rabbit + biot-rabbit, and rabbit + biot-rabbit. As a starting point, we evaluated the 4 Abs for their ability to recognize glycosylated and nonglycosylated BAFF.

ELISA PARAMETERS

Microplates were coated overnight at 4 °C with 3 mg/L per well of anti-BAFF Ab dilutions in carbonate-bicarbonate buffer. We performed 3 washes with PBST in between every 2 assay stages. The wells were saturated with a blocking agent for 3 h and flooded with 100 mL serial dilutions of human rBAFF (Peprotech) or with test sera in 1% BSA-supplemented PBST (PBST/BSA).

After 2-h incubation at 37 °C, we dispensed 3 mg/L of anti-BAFF Ab diluted in PBST/BSA into the wells and left the plates at 37 °C for 90 min. After washes, we filled the wells with 0.1 g/L antigoat Ig biot-donkey, antirabbit Ig biot-donkey, or antimouse Ig biot-goat (all from Jackson), depending on the species of the secondary Ab. After 90 min at 37 °C, we added 0.3 mg/L HRP-conjugated streptavidin (Amersham) diluted in PBST/BSA to each well at 37 °C. The reader was blanked with 0.2% BSA-supplemented PBST. We evaluated Polysorp™ and Maxisorp™ 96-well plates (Nunc) and the polystyrene Costar™ plates (Corning) as microplates in this study. We also determined the effectiveness of 2% BSA, 0.6% gelatin, and 1% casein as blocking agents. We randomly selected another 3 serum samples from the SS patient cohort to calculate the intraassay and interassay CVs with 0.6, 2.5, and 10 μg/L rBAFF.

COMMERCIAL ELISA KITS

We purchased the Quantikine™ (R & D Systems) and APO-54N-020™ ELISA kits (Apotech) for comparison purposes. The former kit is an ELISA pairing the 137134 anti-BAFF mAb with a goat HRP-conjugated anti-BAFF pAb, whereas the latter is based on the combination of an unknown capture anti-BAFF mouse mAb with an unknown detection anti-BAFF rat mAb, plus HRP-conjugated goat antirat Ig pAb.

RF DEPLETION

We tested RFs using the latex test (Fumouze). When present, they were removed to prevent interactions with IgG in the ELISA. Each anti-BAFF mouse IgG1-containing well was paralleled with another well coated with an irrelevant mouse IgG1 *(26)*. The absorbance values in control wells were subtracted from the corresponding absorbances for the anti-BAFF-coated wells. In a second method *(9)*, 1 vol of serum was incubated with another of rabbit IgG-coated Sepharose 4B beads (Pharmacia). After 60 min, the suspension was centrifuged and the supernatant collected. This was repeated 3 times, and the absence of RF in the supernatant was validated by a negative latex test.

Table 1. Unconjugated monoclonal and polyclonal anti-BAFF Abs used in the study.

Ab	Specificity	Immunogen	Origin	Isotype
Monoclonal				
M64	Human	rBAFF	Mouse	IgG1
137314	Human	rBAFF	Mouse	IgG1
Polyclonal				
Rabbit	Human and mouse	aa 254–269	Rabbit	IgG
Goat	Human	rBAFF	Goat	IgG

SOURCE OF SERA

Sera were collected from 68 normal volunteers and 140 patients referred to our hospital and subsequently shown to fulfill the criteria for RA in 28 cases *(27)*, for SLE in 44 cases *(28)*, or for SS in 58 cases *(29)*. In addition, we assessed BAFF in saliva samples from 15 patients with SS with periodontal disease and 15 patients with xerostomia without SS, as well as in SFs from 10 of the previous 28 patients with RA, 8 patients with ankylosing spondylitis, and 9 patients with osteoarthritis. No exclusions were made on any basis other than inability to give informed consent. Informed consent was obtained, and the study was approved by the Institutional Review Board at the Brest University.

STATISTICAL ANALYSIS

All triplicate data were expressed as mean (SD). We calculated percent recovery and all mean absorbance SD and % CVs for intraassay and interassay comparisons using Microsoft Excel.

A P value <0.05 was considered statistically significant. The uncorrected P values were provided along with the effect of the Bonferroni correction whenever that correction would remove statistical significance at the $P < 0.05$ level. We calculated correlations using Spearman's rank correlation and made comparisons using the Mann–Whitney U-test for unpaired data.

Results

RELEVANCE OF GLYCOSYLATION OF BAFF

On the basis that the coating Abs must recognize all possible forms of the protein being assessed, selection of a capture Ab that binds most forms of BAFF was a prerequisite step toward the development of a reliable ELISA. For this purpose, WB was used for testing the available anti-BAFF Abs. Sera from SS patients were taken as the source of multiple forms of BAFF. Given that most of the anti-BAFF Abs had been raised against rBAFF (Table 1), our prediction was that they would bind to this nonglycosylated immunogen, but not to its glycosylated counterpart.

Contrary to expectations (Fig. 1A), M64 bound not only to nonglycosylated or deglycosylated BAFF, but also to glycosylated BAFF. Even worse, 137314 recognized exclusively the glycosylated form of rBAFF. This method was previously validated in our laboratory for its ability to *N*-deglycosylate IgA *(25)*. Here, there was a shift from 28 to 21 kDa. Presumably, the interval from 21-kDa to 17-kDa rBAFF reveals the presence of *O*-glycosylation sites.

In contrast, the 2 pAbs bound to both forms of soluble BAFF. Interestingly, rabbit was raised against a synthetic peptide, and hence was capable of recognizing linear sequences of BAFF, irrespective of its glycosylation status. In addition, there was a weak 23-kDa band, possibly due to recognition of residual IgG light chain, as suggested by others *(30)*. Ultimately, goat appeared to be the most efficient in binding the 2 forms of BAFF.

These paradoxical findings were verified (Fig. 1B) by WB analysis of glycosylated pBAFF and its form deglycosylated by TFMS. The mobility shift of RNase from 15.0 to 13.5 kDa proved that this deglycosylation was effective. On the other hand (Fig. 1C), neither the mAbs nor the pAbs bound to APRIL. Based on these observations, the pAb format was preferred to the mAb format to capture BAFF.

BINDING CHARACTERISTICS OF THE Abs FOR THE ELISA

The microtiter plates were Polysorp, and BSA was used as a blocking agent. Checkerboard combinations helped identify the best compromise between adequate sensitivity and specificity for the capture and the detection Abs at 3 mg/L. Because of the low quantity of pBAFF, it could not be used in the ensuing experiments. Although desirable, it would be more difficult to use pBAFF on a routine BAFF, since the molecule is fragile. Both pAbs were good as capture reagents and functioned successively with each of the 2 mAbs added as detection reagents. Generally, poor sensitivity was found for M64 and a high background for 137314, using either the rabbit (Fig. 2A) or the goat pAb (Fig. 2B) as the coating reagent.

Each of the 2 capture pAbs worked well with the other pAb as a detector, yet there was a loss of sensitivity. The capture rabbit pAbs with detection biot-goat were poor in retaining BAFF on the plates, possibly because of the need to be absorbed before being made commercially acceptable *(32)*.

Of the remaining homologous 2 pairs, the combination of 2 rabbit pAbs was relatively insensitive. In contrast, the combination of 2 goat pAbs not only proved sensitive, but also yielded sigmoid-shaped curves with a linear segment (dotted area in Fig. 2B).

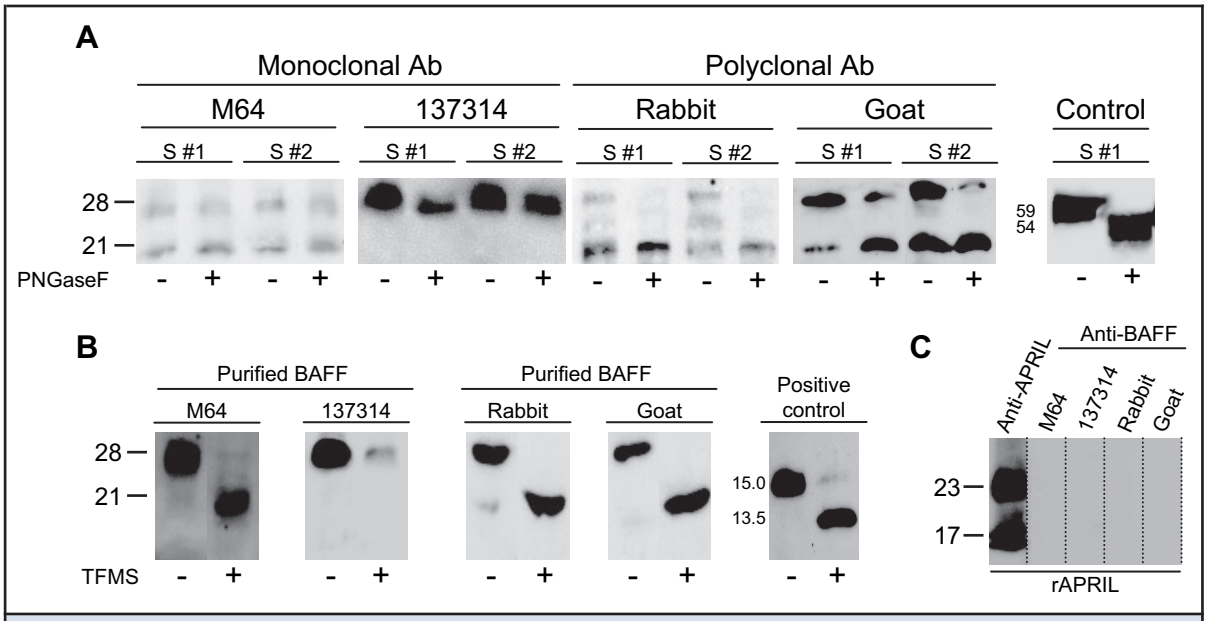

Fig. 1. Binding of 2 anti-BAFF mAbs, M64 and 137314, and 2 polyclonal Abs, rabbit and goat, to BAFF.

(A), BAFF present in the serum of 2 patients with Sjögren syndrome examined before (S #1) and after (S #2) deglycosylation with *N*-glycosidase F (PNGaseF). IgA was the positive control. (B), BAFF affinity-purified from the supernatant of U937 cells in culture and probed with the 4 Abs before and after deglycosylation with trifluoromethanesulfanic acid (TFMS). RNase was the positive control. (C), APRIL tested with the 4 Abs, using an anti-APRIL mAb as a control.

Consequently, this latest pair of capture goat and detection biot-goat was used thereafter in developing the ELISA. This ELISA was also used to confirm (Fig. 2C) that the anti-BAFF pAbs did not recognize APRIL.

REFINEMENT OF THE TEST

Calibration curves (Fig. 3) showed that the optimal concentration of the capture Ab was 0.5 mg/L with an arbitrarily fixed dose of 0.5 mg/L for the detection biot-Ab (Fig. 3A), whereas the optimal concentration of detection biot-Ab was 0.1 mg/L with an arbitrarily fixed dose of 0.5 mg/L for the capture Ab (Fig. 3B).

We next evaluated the efficiency of different ELISA microplates to retain the goat pAb used for coating. This is reflected by the amount of added BAFF captured by the coating Ab and the consequent

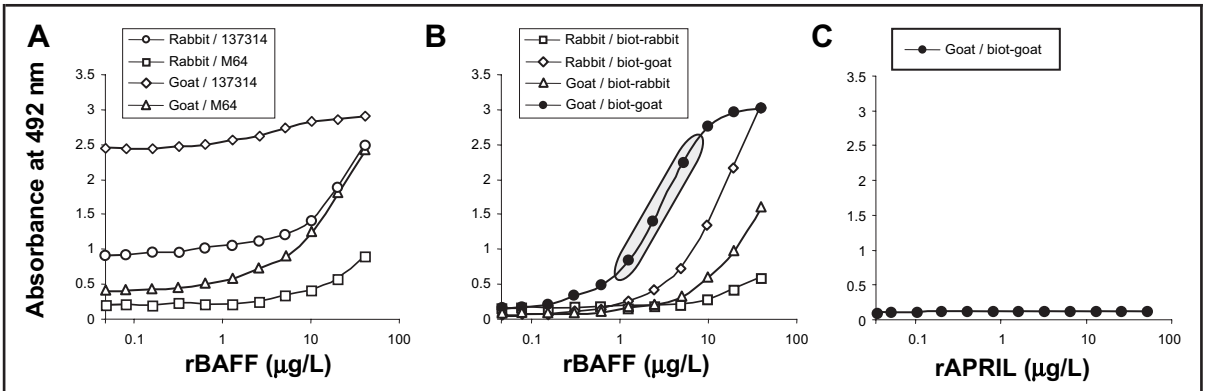

Fig. 2. Dose–effect curves of 8 pairs of antibodies.

The capture Ab is the first member, and the detection Ab the second. (A), Capture pAbs and detection mAbs. (B), Capture pAbs and detection pAbs. (C), Lack of anti-BAFF pAb cross-reactivity with APRIL in the goat pAb/biot-goat pAb assay.

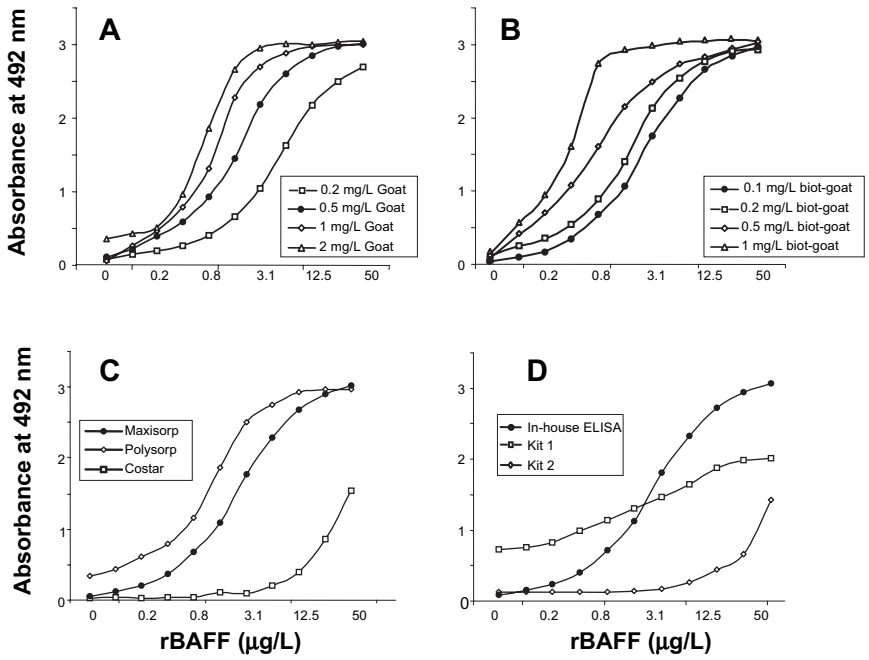

Fig. 3. Refinement of the ELISA.

(A), Determination of the optimal doses of capture goat pAb with the detection biot-goat pAb at 0.5 mg/L. (B), Optimal doses of the detection biot-pAb, with the capture goat pAb at 0.5 mg/L. (C), Comparison of 3 microtiter plates using the pair goat pAb + biot-goat pAb. (D), Comparison of the in-house established ELISA with 2 commercial kits for BAFF.

amount of bound biot-goat pAb (Fig. 3C). There were fewer molecules of BAFF bound by the coating Ab when the Costar plates were used than when using the high-affinity plates. The 2 high-affinity plates (Polysorp and Maxisorp) produced equivalent dose–response curves; the former was selected because of its slightly higher sensitivity.

Finally, different proteins were examined for their ability to quench free sites. Better dose–response curves were obtained with BSA compared with gelatin (increased background) and casein (decreased sensitivity); BSA was therefore considered optimal for the ELISA.

ASSAY IMPRECISION

Intraassay and interassay CVs were calculated by constructing a calibration curve using 0.6, 2.5, and 10 μg/L BAFF, and data were expressed as the ratio of the SD to the mean of 5 measurements. The intraassay CV ranged from 3.5% to 7.5%, and the interassay CV from 4.8% to 9.7% (see Supplemental Table 1, which accompanies the online version of this article at http://www.clinchem.org/content/vol55/issue10). These results also showed the lowest detection limit at 3 SD above the mean of 0, 0.1, 0.2, and 0.4 μg/L rBAFF. The background absorbance range of 0.041–0.071 did not cross over the absorbance range of 0.221–0.311 for 0.1 μg/L rBAFF. This low background reflects the setting of the plate reader. The detection limit was thus confirmed to be 100 ng/L in serum. Detection limits were 111 ng/L in saliva and 30 ng/L in SF.

Albeit less precise, a regression analysis was carried out from the linear parts of the curve by plotting the concentrations of rBAFF in μg/L against the logs of [(absorbance with BAFF) − (absorbance without BAFF)] in the sera. As above, the linear part of the curve extended from 0.04 to 4.0 μg/L.

ELISA VALIDATION

To evaluate the relationship of BAFF and disease, we tested the ELISA by screening sera *(32)* from 68 normal controls, 44 patients with SLE, 28 patients with RA, and 58 patients with SS. The 3 patient groups displayed higher concentrations than the controls (Table 2). We detected salivary BAFF in 15 cases of SS, but not in 15 cases of xerostomia without SS, nor in 10 patients with periodontal disease *(33)*. BAFF was also found in 9 of 10 SFs from RA, 6 of 8 SFs from ankylosing spondylitis, and 0 of 9 SFs from osteoarthritis. Interestingly, as described *(6)*, the concentrations of BAFF in the sera of

Table 2. Concentrations of BAFF in serum, saliva, and SF of patients with SLE, RA, SS, periodontal disease, xerostomia, ankylosing spondylitis, and osteoarthritis.

Sample and population	Samples, n	Mean BAFF concentration (SD), μg/L	Positive, n (%)
Serum			
SLE	44	10.6 (8.5)	23 (52)[a]
RA	28	9.7 (1.5)	5 (18)[b]
SS	58	15.8 (12.9)	35 (51)
Normal	68	4.8 (3.8)	
Saliva			
SS	15	7.4 (2.1)	10 (67)[c]
Periodontal disease	10	2.2 (0.6)	5 (50)
Xerostomia	15	1.0 (0.4)	4 (27)
SF			
RA	10	13.2 (3.5)	9 (90)
Ankylosing spondylitis	8	11.7 (3.9)	6 (75)
Osteoarthritis	9	5.9 (1.1)	0 (0)

[a] $P < 0.001$ and [b] $P = 0.017$ compared with normal controls.
[c] $P = 0.0016$ compared with both groups of disease controls.

9 RA patients correlated with those in their SFs ($P = 0.041$).

We compared the calibration curves for the in-house ELISA with those of 2 commercial kits. The results indicated (Fig. 3D) that our ELISA was suitable for detecting low concentrations of rBAFF. In contrast, the slope of the curve of kit 1 was insufficient to discriminate intermediate values, and the curve generated by kit 2 began at too high concentrations of BAFF to detect low concentrations.

Titers of RF correlated with the absorbances of anti-BAFF Ab–coated wells (Fig. 4A), but not with absorbances of irrelevant IgG–coated wells. This discrepancy suggests that RF does not compete with BAFF for the capture Ab. Had RF influenced the assay, an inverse correlation would have been observed between RF titers and absorbances for irrelevant IgG.

Two experiments confirmed this interpretation. The first (Fig. 4B) consisted in comparing sera without and with preabsorption with rabbit IgG, the second in testing serum samples twice, once in an anti-BAFF–coated well and once in a well coated with irrelevant IgG, and subtracting the background absorbance from the test absorbance. Clearly, RF interfered less in the in-house than in the commercial ELISAs: absorption of RF reduced the test absorbance by only 14.2% (24.0%), demonstrating that the values had not been nonspecifically augmented by serum RF.

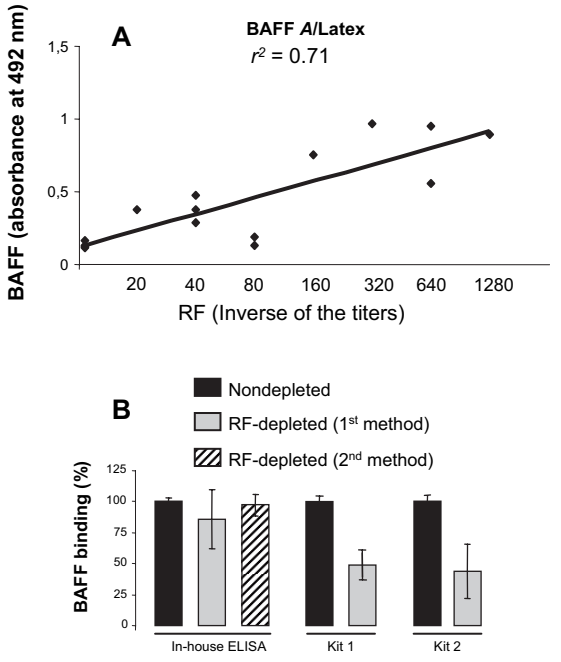

Fig. 4. Interference of serum RF with the anti-BAFF capture antibody coated onto the plate.

(A), Correlation between serum concentrations of BAFF and titers of RF. (B), Percentage of the maximum binding following absorption of RF using 2 methods (see Materials and Methods for technical details). *A*, absorbance.

Discussion

The development of our ELISA for the measurement of BAFF helped provide clues as to why the concentrations of BAFF vary between patients with the same disease using a given assay, and for the same patient using different assays. The success of such a test depends largely on the quality of the Abs selected. In this respect, our most noticeable result was the exclusive binding of 137314 to the glycosylated form of BAFF despite that it had been raised against nonglycosylated BAFF. One explanation is that dissociating conditions of SDS-PAGE destroy the epitopes against which the Abs were produced in response to immunization with 3-dimensional rBAFF. Furthermore, assembly of these molecules into inclusion bodies within *E. coli* might draw together 2 amino acids originally separated, create artificial *N*- or *O*-glycosylation sites in the immunized animal *(34)*, and induce the production of Abs to glycosylated BAFF.

Treatment with *N*-glycanase of the 134-aa 285 domain does not reduce its molecular weight, indicating that the threonine at site 242 is not glycosylated in the

main form of BAFF *(1)*. Alternatively, this site might be accessible in a restricted number of proteins. Assuming that some forms are glycosylated, neoconformational epitopes may be generated by glycosylation at this site. Some pAbs present in the assay might detect such epitopes. This is crucial, as carbohydrates prevent unfolding of globular proteins. The 28-kDa version of BAFF could represent this glycosylated form of the cytokine. Parenthetically, it is not surprising that M64 recognized glycosylated, nonglycosylated BAFF, since we selected it to purify BAFF, and since the manufacturer possibly used the glycosylated form of BAFF to raise the Ab response in the mouse.

Heterogeneity in the concentration of circulating BAFF protein found in SLE patients could arise through different routes *(34)*. The correlation between concentrations of BAFF and disease activity remains modest at best *(35)*. The most tenable explanation is that none of the assays provides sensitive and specific means of measurements, as suggested by the observation that disease activity correlates with the number of transcripts for BAFF in blood cells from SLE patients, but not with the protein concentration *(15)*. It is also possible that the lack of increase in BAFF concentrations in the serum of some SLE patients, notwithstanding the activity of their disease, reflects the diminished release of the cytokine by an increased proportion of Δ3BAFF *(36)*.

Another bias might be an excess of nonglycosylated BAFF at the expense of glycosylated BAFF. The absorbance would thus underestimate the concentration of BAFF, because the capture Ab may mainly recognizes glycosylated BAFF. Previous studies have suggested that changes in *N*-glycan synthesis cause aberrant glycosylation in SLE-prone mice *(37)*. High proportions of heterotrimers of BAFF and APRIL in SLE patients may also cause underestimation of BAFF *(20)*.

To conclude, the measurement of BAFF is useful for following progress of diseases. Differences in the distribution of the forms of BAFF denote the potential of SLE patients to respond to, or to resist, BAFF antagonist therapy *(38)*. Thus reliable indicators for predicting this behavior *(39)* are becoming increasingly important.

Author Contributions: *All authors confirmed they have contributed to the intellectual content of this paper and have met the following 3 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; and (c) final approval of the published article.*

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