Immunology 1993 **80** 191–196

Naturally occurring anti-band 3 antibodies have a unique affinity for C3

H. U. LUTZ, M. NATER & P. STAMMLER
Laboratory for Biochemistry, Swiss Federal Institute of Technology, ETH-Zentrum, Zurich, Switzerland

Accepted for publication 26 May 1993

SUMMARY

Naturally occurring anti-band 3 antibodies appear to mediate opsonization of oxidatively stressed and *in vivo* aged red cells. Their low concentration in plasma (< 100 ng/ml) and weak affinity (estimated association constant, $5–7 \times 10^6$ l/mol) contrasted with their biological efficiency. In compensating for their inadequate properties they have an affinity for C3 at a site independent of the antigen binding domain, with an estimated association constant of $2–3 \times 10^5$ l/mol. Though weak, their binding to C3 was about 100 times higher than that of whole IgG, which is known to have an affinity for C3. The affinity for C3 may render these antibodies preferred targets of the short-lived nascent C3b and result in a preferential C3b–anti-band 3 complex formation. C3b–IgG complexes represent the best opsonins and can nucleate alternative complement pathway C3 convertases by which opsonization is further enhanced.

INTRODUCTION

Naturally occurring autoantibodies (NOA)[1] directed against a large number of self antigens have been found in sera of normal animal and human.[1,2] They are—where investigated—translation copies of the germ line Ig genes with none or very few mutations.[3] Several hypotheses have been raised to explain the biological role of NOA in tissue homeostasis,[4–7] in prevention of autoimmune diseases,[8] and idiotypic/anti-idiotypic network regulation.[9] Partial evidence has been provided for each one of the hypotheses, but a thorough understanding of the role of NOA has remained elusive. The difficulties in understanding the role of NOA are manifold: (1) avid natural antibodies to exposed surface antigens and soluble components are suppressed early in development;[10] (2) those that persist have generally a low affinity, being about 1–3 orders of magnitude lower than those of induced antibodies; (3) many NOA are poly-[1] or bispecific.[11] Thus, their functional relevance has been questioned and only a small number of studies has been carried out with purified NOA. Anti-band 3 antibodies that bind to band 3 protein, the anion transport protein, of human red blood cells (abbreviated as 'anti-band 3 antibodies') represent an example of NOA for which considerable evidence has been presented to support a tissue homeostatic role (reviewed in ref. 12), but little information has been given to explain their functionality. Anti-band 3 NOA were purified from IgG of individuals and pooled IgG (Sandoglobulin^R) by immunoadsorption on immobilized band 3 protein.[7] Their existence in human plasma has been confirmed by applying ELISA,[13] and crossed immune electrophoresis.[14] Anti-band 3 antibodies exist in serum at concentrations of 50–100 ng/ml.[7] Despite their low concentration and weak affinity, these antibodies were preferentially associated with dense (senescent) red blood cells in amounts of up to 200 molecules per cell, which apparently were sufficient for enhanced *in vitro* phagocytosis.[5,15,16] This is in great contrast to induced anti-red cell antibodies, of which thousands or more molecules were required per cell to induce an efficient phagocytosis.[17] Thus, anti-band 3 antibodies appear to have a remarkable but unexplained efficiency. Their increased binding to senescent and oxidatively stressed red blood cells appears to be due to antigen oligomerization,[7,18,19] which results in a firm binding at a concentration where a monovalently bound antibody would dissociate. Anti-band 3 antibodies further gain functionality by an efficient complement deposition, since they stimulated alternative complement pathway C3b deposition in a dose-dependent way and thereby enhanced phagocytosis.[19,20] A mechanism by which such antibodies stimulate alternative complement pathway C3b deposition has been postulated (reviewed in ref. 12). The proposed mechanism suggests that those IgG molecules that stimulate the alternative pathway have an affinity for C3 which exceeds that of other IgG molecules. In an attempt to elucidate the mechanisms by which these naturally occurring anti-band 3 antibodies gain functionality, we have studied binding of anti-band 3 NOA to C3.

Abbreviations: C3b–IgG, C3b that is covalently linked to IgG; HSA, human serum albumin; IgG$^-$, human IgG depleted of anti-idiotypic antibodies, anti-spectrin, and anti-band 3 antibodies; NOA, naturally occurring antibodies; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethylsulphonylfluoride.

Correspondence: H. U. Lutz, Laboratory for Biochemistry, Swiss Federal Institute of Technology (ETH), ETH-Zentrum, CH 8092 Zurich, Switzerland.

MATERIALS AND METHODS

Naturally occurring anti-band 3

Standard protocol. Naturally occurring anti-band 3 antibodies[7] were purified from human IgG as described, with the

following modifications. Pooled human IgG (Sandoglobulin^R, SRC, Berne, Switzerland) (36 g) was dissolved in 300 ml buffer L (100 mM NaCl, 10 mM phosphate, 1 mM $MgCl_2$, 50 μg/ml PMSF, 0·08% sodium azide, 0·05% Nonidet NP-40, pH 7·4), dialysed against buffer L, and diluted before use to 25 mg/ml IgG. This solution was depleted of anti-idiotypic antibodies[21] by passing through a column containing immobilized, heat-aggregated IgG. The flow through was pumped at room temperature through a column containing immobilized spectrin dimer and subsequently through one containing band 3 protein that was purified according to ref. 22. A sample of the flow through was dialysed against phosphate-buffered saline (PBS) containing 0·08% sodium azide and used as control IgG, depleted of anti-band 3 antibodies (IgG^-). The columns were washed overnight with buffer L containing 0·01% NP-40. Bound antibodies were eluted with 1 M NaCl, 0·1 M glycine, 0·01% NP-40 (pH 2·7), neutralized immediately and dialysed against PBS containing 0·08% sodium azide. Purified antibodies were concentrated in Centricon 30 microconcentrators (Amicon, Beverley, MA) and the IgG concentration was determined.[23] The columns were reused at least 10 times. Recoveries and immunoblots with labelled antibodies served as a quality control.

Experimental procedures. Where indicated, IgG was subjected to different purification protocols. After passing through the column containing spectrin it was either passed through a column containing immobilized C3 or one to which a mixture of equal milligrams of band 3 protein and C3 was immobilized [anti-(band 3+C3)]. Repurified anti-band 3 antibodies were obtained by dialysing them against buffer L and passing over a new column containing band 3 protein. The columns were washed and eluted as given in the standard procedure, except that repurified anti-band 3 antibodies were eluted in a batch procedure to minimize dilution.

Complement component C3

C3 was purified[24,25] from fresh human plasma. C3 was dialysed against 150 mM NaCl, 10 mM phosphate, 2 mM EDTA, 50 μg/ml phenylmethylsulphonylfluoride (PMSF), 0·01% sodium azide (pH 7·4) and was filtered through 0·2-μm filters into sterile tubes. C3 was stored at 1–2 mg/ml in aliquots at 4°. Freshly purified C3 did not contain α'C3b in its reduced form and could be activated by trypsin.[26] It was free from IgG as judged from immunoblots.

Iodination of antibodies

NOA and preparations of IgG^- were labelled with ^{125}I-iodine using chloramine T as an oxidant.[27] The reaction was stopped with metabisulphite and the proteins were gel-filtered over Sephadex G75 in PBS containing 1 mM EDTA and 1 mM NaI. The columns and the collecting tubes were pre-rinsed with PBS containing 1% gelatin. Labelled antibodies were frozen in small aliquots. The specific activities were estimated from the amount of iodinated material and by assuming an average recovery of 50% of the iodinated material. This recovery was determined beforehand in experiments using whole IgG. In experiments where binding of anti-band 3 antibodies and that of IgG^- were compared, both samples were ^{125}I-iodinated in parallel and thus the error in specific activities was less than 10%. They ranged from 10 to 27×10^6 c.p.m. per μg protein.

Radioimmune assays

Chemobond plates were purchased from ANAWA AG, Wangen ZH, Switzerland. They were rinsed with distilled water and coated with either band 3 protein or C3 (20 μg/ml each) at neutral pH by trapping imines with cyanoborohydride following the protocol outlined previously.[28] In the case of band 3 protein, the coating solution contained 0·1% Triton X-100. Unreacted aldehydes were reduced and coated plates were washed and stored at 4° with the wells completely filled with wash buffer. Labelled antibodies were added to wells in a total volume of 150 μl in RIA-buffer (500 mM NaCl, 20 mM Tris, 0·08% sodium azide, pH 7·4) supplemented with 1% gelatin and 0·04% Triton X-100, if not otherwise indicated. Labelled antibodies were thawed once, resuspended in RIA buffer, and precentrifuged for 5 min in an Eppendorf centrifuge. Other additions [50 mg/ml human serum albumin (HSA) (albumin 20%, from the Central Laboratory of Blood Transfusion Services, Berne, Switzerland) where indicated, the given concentrations of C3 or whole IgG] were extensively dialysed against RIA buffer. Band 3 protein was dialysed against RIA buffer containing 0·04% Triton. Mixtures were prepared as follows: labelled antibodies and the various additions were mixed in an Eppendorf tube with 30 μl of warmed RIA buffer containing 5% gelatin and 0·2% Triton X-100, except in case of band 3 protein, where the RIA buffer was supplemented with 5% gelatin and the Triton X-100 concentration adjusted to a final concentration of 0·04%. Triplicates were delivered from this mixture to wells, if not otherwise indicated. Plates were covered and agitated at room temperature overnight on a Vary Shaker (Dynatech, Embrach, Switzerland). Plates were washed three times with RIA buffer supplemented with 0·04% Triton X-100 and 1% gelatin, three times with 0·9% NaCl, 20 mM Tris, 0·08% sodium azide (pH 7·4) and three times with water. Bound radioactivity per well was determined using a MR 480 gamma-counter (Kontron, Zurich, Switzerland). Antibody binding is given in pg or ng/well. The extent of binding was calculated from the amount of bound radioactivity with background subtracted and by using the specific activity of the added antibodies. In case the samples were supplemented with increasing concentrations of whole IgG that contained unlabelled anti-band 3 antibodies, the specific activities of the antibodies were corrected for the serum dilution, assuming an average content of anti-band 3 of 50 ng in 15 mg IgG, as determined earlier.[7] When antibody binding was corrected for that of any IgG, antibody and IgG^- preparations were added at the same concentration and total radioactivity, bound radioactivity was determined, expressed in pg/well for anti-band 3 antibodies and IgG^-, and the difference between these values was plotted.

Membranes from human red blood cells

Human blood, type O Rh^+, was collected in heparin and processed within 1 hr after collection. Red blood cells were freed from white cells essentially according to ref. 29 and as modified in ref. 30. Membranes from washed cells were prepared by hypotonic lysis and serine proteases inactivated as outlined elsewhere.[30]

SDS–PAGE

Samples kept in 1% SDS and 5 mM N-ethylmaleimide were thawed and mixed with electrophoresis sample buffer containing 40 mM dithiothreitol where indicated. Denaturation was for

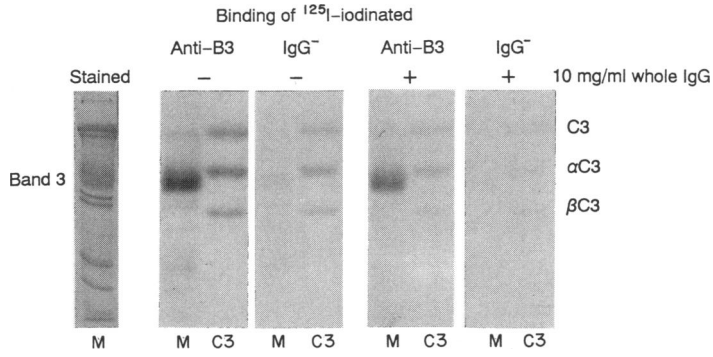

Figure 1. Binding of ^{125}I-iodinated anti-band 3 antibodies (anti-B3) and of IgG$^-$ to polypeptides from red blood cell membranes and to C3. Membrane proteins (M) (7·5 μg) and 2·5 μg of reduced and unreduced C3 (C3) were denatured for 30 min at 37°, run on an SDS–PAGE, and blotted to nitro-cellulose. Blots were incubated with either 66 ng/ml of labelled anti-band 3 antibodies or IgG$^-$ containing 400,000 c.p.m./ml. Incubation mixtures were supplemented with 10 mg/ml whole human IgG, where indicated (+). The figure shows autoradiographs of blots and a stained blot from membranes. Similar results were found with several independent antibody preparations. No significant difference was observed whether blotted material was denatured for 3 min at 100° or for 30 min at 37°.

30 min at 37° or for 3 min in a boiling water bath, as indicated. All samples were then alkylated by adding 50 mM N-ethylmaleimide. Electrophoresis on SDS–PAGE was performed on minigels using a modified version of the Neville[31] gel system.[32] Reduced molecular mass standards (Bio-Rad, Richmond CA) were loaded where appropriate. The running gel contained 8% or 10% acrylamide and 2·7% bisacrylamide. Gels were either electroblotted or stained, dried and exposed to X-Omat Xray films (Kodak, Lausanne, Switzerland).

Immunoblotting

Red cell membrane polypeptides, reduced or unreduced C3, or a mixture of both made after alkylation, were electrophoretically separated. Gels were blotted onto nitro-cellulose as outlined elsewhere.[7] Blots were preincubated for 1 hr at 37° to block unspecific binding of labelled antibodies by adding buffer R (0·9% NaCl, 20 mM Tris, 0·08% sodium azide, 50 μg/ml PMSF, pH 7·4) that was supplemented with 2% gelatin. Blots were incubated overnight at room temperature with the given labelled antibodies in buffer R supplemented with 1% gelatin, 0·04% Triton X-100 and the additions indicated. Blots were washed three times with 1% gelatin and 0·04% Triton X-100 in R buffer, then three times with the same buffer and three times with buffer R alone by alternating between the two types of washing solutions. Blots were dried and autoradiographs from dried blots are shown.

RESULTS

Anti-band 3 antibodies appeared as monospecific NOA when probed on blots from human red cell membrane polypeptides, since they bound almost exclusively to band 3 protein (Fig. 1, M). Despite this specificity they also bound to blotted C3 in its unreduced and reduced form (Fig. 1). Their binding was $2{\cdot}4 \pm 0.37$ times ($n=4$ independent blots) higher to α than to βC3, and bound antibody increased 16–20-fold when its concentration was raised from 50 to 3000 ng/ml (n=2). Some binding to C3 appeared as either non-specific or common to all IgG molecules, since labelled IgG depleted of anti-band 3 antibodies (IgG$^-$) no longer bound to band 3 protein, but maintained a low binding to C3 and its subunits (Fig. 1, IgG$^-$).

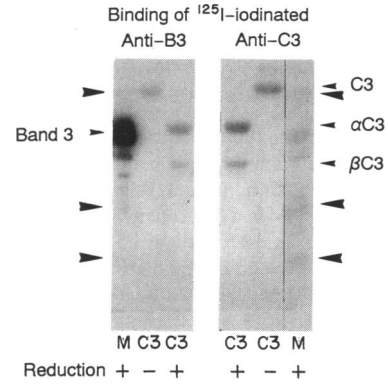

Figure 2. Binding of ^{125}I-iodinated anti-band 3 antibodies (anti-B3) and of IgG purified on immobilized C3 (anti-C3) to membrane polypeptides and C3 on blots. Membrane proteins (M) (5 μg) and 2 μg of reduced or unreduced C3 were denatured for 30 min at 37°, run on SDS–PAGE, and blotted to nitro-cellulose. Blots were incubated with 20 ng/ml of either antibody containing 1 and $0{\cdot}92 \times 10^6$ c.p.m./ml, respectively. Autoradiographs are shown from one out of several blots performed from two anti-C3 antibody preparations. The three unlabelled, heavy arrows point to minor polypeptides originating from band 3 protein.

Its binding to C3 was suppressed by a 10^5-fold excess of unlabelled, whole human IgG (Fig. 1, IgG$^-$ + whole IgG). On the other hand, addition of the same excess of whole human IgG to labelled anti-band 3 antibodies lowered, but neither eliminated their binding to band 3 protein nor to αC3. Thus, anti-band 3 antibodies specifically bound to their antigen, weakly to αC3 and even less to intact C3 at their physiological concentration in the presence of a 10^5-fold excess of other IgG molecules. The same results were obtained with either 0·04% Triton X-100 or 0·1% Tween 20 in the binding buffer.

Binding of anti-band 3 antibodies to C3 did not originate from contaminating antibodies, since repurification on band 3 protein did not eliminate their binding to C3 (not shown). Likewise, IgG purified on immobilized C3 bound to C3 and among red cell polypeptides primarily to band 3 protein and some other polypeptides. Three of them probably originated from band 3 protein and also reacted with purified anti-band 3 antibodies (oligomer and two fragments, see heavy arrows in

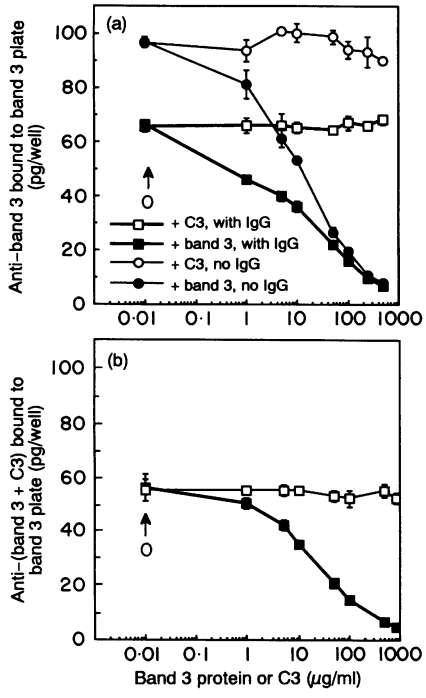

Figure 3. Binding of ^{125}I-iodinated anti-band 3 (a) and of anti-(band 3+C3) (b) to covalently immobilized band 3 protein. Labelled antibodies were mixed with 15 mg/ml unlabelled, whole human IgG and incubated with immobilized band 3 protein in the presence of increasing concentrations of either band 3 protein or C3 as indicated. In the case of anti-band 3 antibodies purified by standard procedures (a), binding to band 3 protein was also studied in the absence of whole IgG. Anti-band 3 antibodies were applied at 133 ng/ml, containing $4{\cdot}36 \times 10^6$ c.p.m./ml, anti-(band 3+ C3) antibodies were applied at 250 ng/ml, containing $4{\cdot}4 \times 10^6$ c.p.m./ml. Averaged data from triplicates are shown ± 1 SD. Where SD bars are not visible, the SD value was smaller than the symbols. Similar results were obtained with two different antibody preparations.

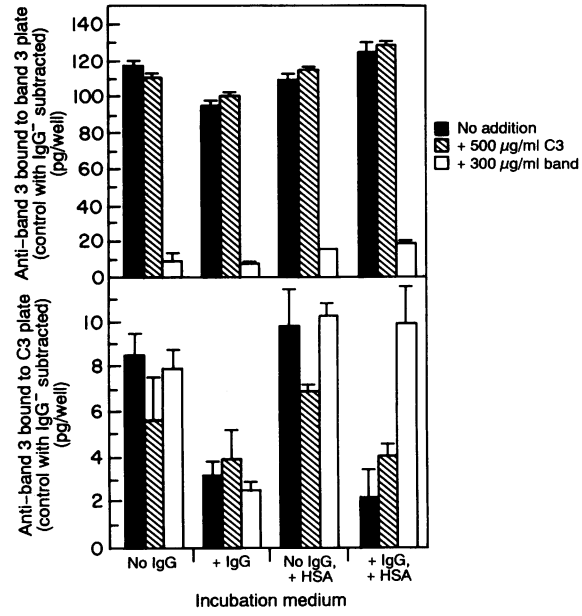

Figure 4. Anti-band 3 binding to immobilized band 3 protein and C3 as a function of the incubation medium. Anti-band 3 antibodies and, in control assays, IgG$^-$ preparations were applied at 150 ng/ml, containing 4×10^6 and $3{\cdot}7 \times 10^6$ c.p.m./ml, respectively, to immobilized band 3 protein or C3. The incubation medium was varied and contained, besides 1% gelatin in RIA-buffer, no addition (no IgG); 15 mg/ml whole, unlabelled IgG (+ IgG); 50 mg/ml HSA (no IgG,+ HSA); or 15 mg/ml whole, unlabelled IgG and 50 mg/ml HSA (+IgG, + HSA). The media were further supplemented with 500 μg/ml C3 or 300 μg/ml band 3 protein where indicated. The anti-band 3 specific binding was determined by subtracting the extent of binding observed with IgG$^-$. Averaged data from triplicates are shown +1 SD.

Fig. 2). Thus, the ability to interact with C3 represented a unique property of anti-band 3 antibodies.

Anti-band 3 antibody binding to C3 could be mediated by the antigen-binding site, if these antibodies had a bispecificity, as is common among NOA.[11] Binding could, however, be by an allotypic IgG domain within the Fd fragment or by sites within the framework of Fab, outside the antigen-binding site. To characterize their properties, binding was studied using a RIA to covalently immobilized band 3 protein or C3. Anti-band 3 antibodies bound specifically to band 3 protein, since fluid phase band 3 protein inhibited binding completely at concentrations exceeding 200 μg/ml and to 50% by 15–20 μg/ml (Fig. 3). Competitive inhibition was equally effective whether measured in the presence or absence of physiological concentrations of IgG (Fig. 3a). C3, however, did not inhibit their binding to immobilized band 3 protein at all. Similar results were obtained with anti-band 3 antibodies that were affinity purified on a 1/1 mixture of band 3 protein and C3 [anti-(band 3 + C3)] (Fig. 3b). Furthermore, C3 (500 μg/ml) did not inhibit antibody binding to band 3 protein in several incubation media, in which fluid phase band 3 protein was inhibitory (Fig. 4). These results suggest independent binding sites for band 3 protein and C3.

Anti-band 3 antibody binding to covalently immobilized C3 was somewhat more difficult to determine, since IgG$^-$ also bound to C3, primarily in the absence of unlabelled IgG (see Fig. 1). Thus, binding of anti-band 3 antibodies was corrected for that of any IgG, by subtracting the extent of binding determined for labelled IgG$^-$ (Fig. 4). Anti-band 3 binding to C3 was about ten times lower to C3 than to band 3 protein in several incubation media. Fluid phase C3 at 500 μg/ml partially inhibited their binding to C3 by 20–25% in the absence of whole IgG. Band 3 protein at 300 μg/ml did not inhibit anti-band 3 binding to C3 in any of the incubation media (Fig. 4). This again suggests that the binding sites for band 3 protein and C3 differ from each other. Whole human IgG added at an excess of 10^5-fold appeared to compete with anti-band 3 antibodies for C3, since it lowered anti-band 3 binding to C3 by 60 to 75% (Fig. 4, + IgG). Most interestingly, fluid phase band 3 protein stabilized anti-band 3 binding to C3, when human serum albumin was present, but not when it was absent (Fig. 4). In fact, the addition of 300 μg/ml of band 3 protein overcame the inhibitory effect of IgG and enhanced anti-band 3 binding to C3 by 2–3-fold compared to the assay without band 3 protein.

Binding of anti-band 3 antibodies and whole IgG to C3 was further differentiated by studying antibody binding in the presence of increasing concentrations of whole human IgG (Fig. 5). Fluid phase C3 (0·1 to 1·5 mg/ml) inhibited anti-band 3 antibody binding to immobilized C3 significantly at IgG/antibody ratios of up to 10^4, but remained ineffective at and above a ratio of 10^5 (Fig. 5). This suggests that anti-band 3 binding to C3 was considerably better than that of the remaining

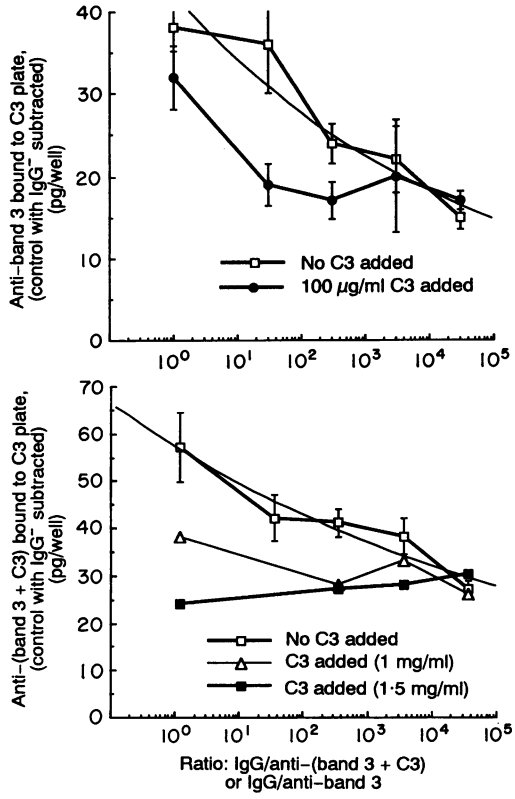

Figure 5. Binding of labelled anti-band 3 and anti-(band 3 + C3) antibodies to immobilized C3 in the presence of increasing concentrations of whole, unlabelled IgG and fluid phase C3. Antibodies or IgG^- were added at 100 ng/ml, containing $2{\cdot}93 \times 10^6$ c.p.m./ml, to immobilized C3 in the presence or absence of 0·1–1·5 mg/ml C3 and increasing concentrations of whole, unlabelled IgG. Binding was corrected for that observed with IgG^- and is plotted against a ratio of the total IgG concentration over that of labelled antibodies. Averaged data from triplicates are shown ± 1 SD where indicated. Results from samples with high concentrations of C3 were averaged from duplicates. Binding of antibodies in the absence of C3 is also shown in form of a regression curve (fine lines). Similar results were obtained in the absence of detergent and with two independent antibody preparations.

IgG molecules. The regression curves show an almost linear decrease with increasing IgG/antibody ratios. Maximum inhibition required 1·5 mg/ml C3 and the specific binding to C3 reached 50% of its original value at an IgG/anti-band 3 ratio of 10^2. Thus, the affinity of anti-band 3 antibodies for C3 was about 10^2 times that of whole IgG.

DISCUSSION

Binding data establish the presence of a unique binding site for C3 on anti-band 3 NOA. The data cannot define a C3 binding site in molecular terms, since they originated from an affinity-purified fraction of pooled, human IgG rather than an IgG subclass.

Anti-band 3 antibodies were mono- rather than polyspecific and bound almost exclusively to band 3 protein among red cell polypeptides. Unlike anti-cardiolipin NOA[11] they were not bi-specific via Fab, since C3 did not compete for their Fab-dependent binding to band 3 protein. Likewise, fluid phase band 3 protein did not inhibit their binding to immobilized C3. Instead band 3 protein even increased their binding to C3 by 2–3-fold in the presence of physiological concentrations of IgG and human serum albumin. This finding strongly suggests independent binding sites and is best explained by assuming that fluid phase band 3 protein stabilized anti-band 3 antibodies loosely associated with C3 in the presence of human serum albumin. Thus, the C3 binding site is most likely located outside the antigen-binding domain, presumably within the Fd region or the framework portion of the Fab.

The binding of anti-band 3 antibodies to C3 was quantitatively and qualitatively different from that of other IgG molecules (IgG^-), since anti-band 3 antibodies bound preferentially to αC3 and their binding to αC3 was lowered but not eliminated by a 10^5-fold excess of whole IgG. It appears as if all IgG have a weak affinity for both polypeptides of C3.[33] In addition, purified anti-band 3 antibodies have an extra affinity for αC3 and less for intact C3. Anti-band 3 binding to immobilized C3 was to native C3, since fluid phase C3, which contained no noticeable C3b, effectively inhibited their binding to immobilized C3. Their estimated association constant was 2×10^5 l/mol for C3 and $5\text{–}7 \times 10^6$ l/mol for band 3 protein.

The affinity for native C3 may render anti-band 3 antibodies preferred targets of nascent C3b and may result in a preferential formation of C3b–anti-band 3 complexes. A preferred generation of C3b–IgG complexes provides potency, because C3b–IgG complexes (1) represent the best opsonins, when generated on antigen-bound IgG;[34] and (2) nucleate alternative complement C3 convertases 30 times better than free C3b.[35] The reason for this is that C3b covalently bound to IgG is protected from inactivation by factor H- and I-dependent proteolysis.[35] Their affinity was only 10^2 times higher than that of the bulk IgG, but this increment may be sufficient to render these antibodies functional at low concentrations and with low affinity for their antigen. Accordingly, they stimulated C3b deposition to oxidatively stressed red blood cells and enhanced their phagocytosis.[19]

ACKNOWLEDGMENTS

We thank Professor H. Heiniger and Dr P. Späth from the Central Laboratory of the Blood Transfusion Service, SRC, Berne for the large supply of outdated human IgG and of albumin. This work was supported by grants to H. U. Lutz from the Swiss National Science Foundation, Berne (3.211.0-85 and 31-25175.88); the Swiss Federal Institute of Technology (ETH), Zurich (0.330.089.37/6); and an award from the Central Laboratory of the Blood Transfusion Service, SRC, Berne.

REFERENCES

1. Avrameas S. (1991) Natural autoantibodies—from horror autotoxicus to gnothi seauton. *Immunol. Today*, **12**, 154.
2. Tomer Y. & Shoenfeld Y. (1988) The significance of natural autoantibodies. *Immunol. Invest.* **17**, 389.
3. Baccala R., Quang T.V., Gilbert M., Ternynck T. & Avrameas S. (1989) Two murine natural polyreactive autoantibodies are encoded by nonmutated germ-line genes. *Proc. natl. Acad. Sci. U.S.A.* **86**, 4624.
4. Grabar P. (1983) Autoantibodies and the physiological role of immunoglobulins. *Immunol. Today*, **4**, 337.
5. Kay M.M.B. (1975) Mechanism of removal of senescent cells by human macrophages in situ. *Proc. natl. Acad. Sci. U.S.A.* **72**, 3521.
6. Lutz H.U. & Wipf G. (1982) Naturally occurring autoantibodies to skeletal proteins from human red blood cells. *J. Immunol.* **128**, 1695.

7. Lutz H.U., Flepp R. & Stringaro-Wipf G. (1984) Naturally occurring autoantibodies to exoplasmic and cryptic regions of band 3 protein of human red blood cells. *J. Immunol.* **133,** 2610.
8. Cohen I.R. & Cooke A. (1986) Natural autoantibodies might prevent autoimmune disease. *Immunol. Today*, **7,** 363.
9. Coutinho A. (1980) The self-nonself discrimination and the nature and acquisition of the antibody repertoire. *Ann. Immunol. (Inst. Pasteur)*, **131 D,** 236.
10. Miller R.D. & Calkins C.E. (1988) Development of self-tolerance in normal mice. Appearance of suppressor cells that maintain adult self-tolerance follows the neonatal autoantibody response. *J. Immunol.* **141,** 2206.
11. McNeil H.P., Hunt J.E. & Krilis S.A. (1990) New aspects of anticardiolipin antibodies. *Clin. exp. Rheumatol.* **8,** 525.
12. Lutz H.U. (1990) Erythrocyte clearance. In: *Blood Cell Biochemistry, Volume 1, Erythroid Cells* (ed. J. R. Harris), p. 81. Plenum Press, New York and London.
13. Sorette M.P., Galili U. & Clark M.R. (1991) Comparison of serum anti-band-3 and anti-gal antibody binding to density-separated human red blood cells. *Blood*, **77,** 628.
14. Heegaard N.H.H. (1990) Immunochemical characterization of interactions between circulating autologous immunoglobin G and normal human erythrocyte membrane proteins. *Biochim. Biophys. Acta*, **1023,** 239.
15. Kay M.M.B. (1978) Role of physiologic autoantibody in the removal of senescent human red cells. *J. Supramol. Struct.* **9,** 555.
16. Hebbel R.P. & Miller W.J. (1984) Phagocytosis of sickle erythrocytes: immunologic and oxidative determinants of hemolytic anemia. *Blood*, **64,** 733.
17. Ehlenberger A.G. & Nussenzweig V. (1977) The role of membrane receptors for C3b and C3d in phagocytosis. *J. exp. Med.* **145,** 357.
18. Mueller H. & Lutz H.U. (1983) Binding of autologous IgG to human red blood cells before and after ATP-depletion. Selective exposure of binding sites (autoantigens) on spectrin-free vesicles. *Biochim. Biophys. Acta*, **729,** 249.
19. Lutz H.U., Bussolino F., Flepp R., Fasler S., Stammler P., Kazatchkine M.D. & Arese P. (1987) Naturally occurring anti-band 3 antibodies and complement together mediate phagocytosis of oxidatively stressed human red blood cells. *Proc. natl. Acad. Sci. U.S.A.* **84,** 7368.
20. Lutz H.U., Fasler S., Stammler P., Bussolino F. & Arese P. (1988) Naturally occurring anti-band 3 antibodies and complement in phagocytosis of oxidatively-stressed and in clearance of senescent red cells. *Blood Cells*, **14,** 175.
21. Morgan A.C., Rossen R.D., McCormick K.J., Stehlin J.S. & Giovanella B.C. (1982) 'Hidden' cytotoxic antibodies that react with allogeneic cultured fetal and tumor cells contained in soluble immune complexes from normal human serum. *Cancer Res.* **42,** 881.
22. Lukacovic M.F., Feinstein M.B., Sha'afi R.I. & Perrie S. (1981) Purification of stabilized band 3 protein of the human erythrocyte membrane and its reconstitution into liposomes. *Biochemistry*, **20,** 3145.
23. Gee A.P. & Langone J.J. (1981) Immunoassay using ^{125}I- or enzyme-labeled protein A and antigen-coated tubes. *Analyt. Biochem.* **116,** 524.
24. Tack B.F. & Prahl J.W. (1976) Third component of human complement: purification from plasma and physicochemical characterization. *Biochemistry*, **15,** 4513.
25. Hammer C.H., Wirtz G.H., Renfer L., Gresham H.D. & Tack B.F. (1980) Large scale isolation of functionally active components of the human complement system. *J. biol. Chem.* **256,** 3995.
26. Newman, S.L. & Johnston R.B. (1979) Role of binding through C3b and IgG in polymorphonuclear neutrophil function: studies with trypsin-generated C3b. *J. Immunol.* **123,** 1839.
27. Bächi Th., Dorval G., Wigzell H. & Binz H. (1977) Staphylococcal protein A in immunoferritin techniques. *Scand. J. Immunol.* **6,** 241.
28. Lutz H.U., Stammler P. & Fischer E.A. (1990) Covalent binding of detergent-solubilized membrane glycoproteins to 'Chemobond' plates for ELISA. *J. immunol. Meth.* **129,** 211.
29. Beutler E., West C. & Blume K.-G. (1976) The removal of leukocytes and platelets from whole blood. *J. Lab. clin. Med.* **88,** 328.
30. Lutz H.U., Stammler P., Fasler S., Ingold M. & Fehr J. (1992) Density separation of human red blood cells on self forming Percoll^{R} gradients: correlation with cell age. *Biochim. Biophys. Acta*, **1116,** 1.
31. Neville D.M. (1971) Molecular weight determination of protein-dodecyl sulfate complexes by gel electrophoresis in a discontinuous buffer system. *J. biol. Chem.* **246,** 6328.
32. Fasler S., Skvaril F. & Lutz H.U. (1988) Electrophoretic properties of human IgG and its subclasses on sodium dodecyl-sulfate-polyacrylamide gel electrophoresis and immunoblots. *Analyt. Biochem.* **174,** 593.
33. Kulics J., Rajnavölgyi E., Füst G. & Gergely J. (1983) Interaction of C3 and C3b with immunoglobulin G. *Mol. Immunol.* **20,** 805.
34. Fries L.F., Siwik S.A., Malbran A. & Frank M.M. (1987) Phagocytosis of target particles bearing C3b–IgG covalent complexes by human monocytes and polymorphonuclear leucocytes. *Immunology*, **62,** 45.
35. Fries L.F., Gaither T.A., Hammer C.H. & Frank M.M. (1984) C3b covalently bound to IgG demonstrates a reduced rate of inactivation by factors H and I. *J. exp. Med.* **160,** 1640.