Dissection of C1q Capability of Interacting with IgG

TIME-DEPENDENT FORMATION OF A TIGHT AND ONLY PARTLY REVERSIBLE ASSOCIATION

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C1q-bearing immune complexes have been observed in diseases such as rheumatoid arthritis and human immunodeficiency virus infection-associated neuropathy. For the purpose of understanding better the phenomenon of C1q-bearing immune complexes, we investigated the constancy of the C1q-IgG interaction. An enzyme-linked immunosorbert assay was developed in which wells were coated with IgG to mimic antigen-complexed IgG. Serial dilutions of C1q were applied for distinct time intervals, and bound C1q was detected either directly or after exposure to one of several elution buffers. Our results show that a part of C1q attached to IgG forms a tight association that is not reversible under treatment with buffers containing usually protein-protein interaction-dissociating reagents such as 3 M NaCl, 5 M urea, sodium dodecyl sulfate, or β-mercaptoethanol. The formation of the highly stable C1q-IgG complex was found to be time-, temperature-, and pH-dependent and to proceed with bound C1q even in the absence of free C1q in the supernatant. In ligand blotting experiments we demonstrate for the first time directly that all three chains of C1q can individually bind IgG. Altogether, our results provide a suitable explanation for the formation and persistence of C1q-bearing immune complexes.

The collagen-like C1q molecule is a subcomponent of C1, the first component of complement, and provides a link between the innate immune system, namely the classical complement pathway, and the acquired immunity and some of its most prominent actors, the immunoglobulin classes G and M.

Human C1q is a glycoprotein of about 460 kDa (1). In its electron microscopy image C1q appears as a bunch of tulips, with six globular heads, each connected by a stalk to a central bundle of fibers (2). In the model of Reid and Porter (3), one C1q molecule is composed of 18 polypeptide chains. The chains are of three different types named A, B, and C, of 29, 27, and 23 kDa, respectively. They are linked by disulfide bonds to form six A-B and three C-C dimers. Each of the six individual segments of C1q comprises one chain of each type, which acquire a triple helical structure in the fibrillar region. In contrast, the globular domains appear to be folded to a large extent into β-sheets (4, 5).

Serum C1q is the key molecule for initiation of the classical complement cascade pathway. Its globular domains recognize the Cγ2 domain of IgG or the Cμ3 domain of IgM, especially if these antibodies are complexed with antigen and thus fixed (6–10). However, C1q differentiates among IgG subclasses because it attaches, in terms of binding efficiency, most strongly to IgG3, followed by IgG1, but it hardly associates with IgG2 and does not react with IgG4 (11). Most studies on the interaction between C1q and IgG favor an ionic rather than a hydrophobic nature of the binding (5, 8, 12, 13), but it has been shown that a dipeptide composed of the aromatic amino acids tryptophan and tyrosine, Trp-Tyr, strongly inhibits the association of the two proteins (14).

The attachment site or sites for IgG on the globular regions of C1q have been a subject of investigation for years. It has been reported that the IgG recognition site is mainly of an ionic nature (8) and that the arginine, histidine, and lysine residues might be essential (13, 15). It has been speculated that Glu-198, Asp-200, and Lys-202 of the C1q A chain might fit with a binding site on IgG (12). Marques and colleagues (5) have suggested that C1q provides two recognition sites for IgG. The first is thought to be located in a section ranging from positions 114 to 129 of the B chain with Arg-114 and Arg-129 being the important residues. The second site has been implicated to involve all three C1q protein chains with Arg residues at positions 162 in the A chain, 163 in the B chain, and 156 in the C chain. Histidine residues appeared again to be relevant for the C1q-IgG interaction too. However, all of these suggestions have been deduced from experiments in which distinct amino acid residues of C1q were modified chemically or where cross-linking to heterologous IgG was performed. Jiang et al. (16) suggested that the globular domain of the C chain provides the primary recognition site for IgG, based on Western blot studies. Kishore et al. (17) reported that a recombinant form of the globular region of the B chain immobilized on a microtiter plate binds IgG but not IgM.

Based on site-directed mutagenesis experiments, the binding site for C1q on IgG has been allocated to the fyz β-sheet in the Cγ2 domain, the charged amino acids Glu-318, Lys-320, and Lys-322 being the suggested binding motif (12). A proline residue is present in position 331 in IgG1 but is replaced by a serine in IgG4 and has previously been demonstrated to be, at least in part, responsible for the differential ability of the two isotypes to bind C1q and activate complement (18).

Immune complexes (IC)² bearing C1q (C1qIC) have been observed in patients suffering from diseases such as rheumatoid arthritis and HIV infection-associated neuropathy (19, 20),

* This work was supported by Deutsche Forschungsgemeinschaft Grant SFB 311 D1. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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¹ The abbreviations used are: IC, immune complex(es); C1qIC, C1q-bearing IC; HIV, human immunodeficiency virus; ELISA, enzyme-linked immunosorbent assay; ME, β-mercaptoethanol; DTT, dithiothreitol; BSA, bovine serum albumin; NAC, N-acetylcysteine; hu, human; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; mur, murine; α-, anti-; mAb, monoclonal antibody; gt, goat; rb, rabbit; SSBH, sulfosuccinimidyl-6-(biotinamido) hexanoate; Ab, antibody; mS, millisiemens; ABTS, 2,2’-azino bis(3-ethylbenzthiazoline-6-sulfonic acid); SDSseb, SDS sample buffer; TBS, Tris-buffered saline.

Vol. 272, No. 52, Issue of December 26, pp. 33234–33244, 1997
Printed in U.S.A.
but their physiological role is still a mystery. However, persist-
ence of these protein complexes implies a sufficient stability of
the C1q-IgG association. To understand better the phenome-
on of C1qIC, we investigated the formation and constancy of
the C1q association with IgG.

An enzyme-linked immunosorbent assay (ELISA) was devel-
oped in which wells were coated with IgG to mimic fixed/
agent-complexed IgG. Serial dilutions of purified and bioti-
ylated or unlabeled C1q or serum were applied for distinct
time intervals. Bound C1q was detected via its biotin tag using
an avidin-peroxidase conjugate or antigenically, employing poly-
or mononclonal antibodies, either directly or after exposure
to one of several elution buffers. Interestingly, our results show
that a part of C1q attached to IgG forms a tight association,
which is not reversible upon treatment with buffers contain-
ing usually protein-protein interaction-dissociating reagents
such as 3 M NaCl, 5 M urea, SDS, β-mercaptoethanol (ME), or
dithiothreitol (DTT). The formation of the highly stable C1q-
IgG complex was found to be time-, temperature-, and pH-de-
dendent and proceeded with bound C1q even in the absence of
free C1q in the supernatant.

Because the C1q molecule comprises three disparate but
similar protein chains, we wondered if all three contribute to
the interaction with homologous IgG. Therefore, we performed
ligand blotting experiments with the separated protein chains of
C1q.

Altogether, our results demonstrate directly for the first time
that all three chains of C1q can bind IgG individually and that
the C1q-IgG interaction is strengthened upon time and thus
provides a suitable explanation of the formation and persist-
ence of C1qIC.

EXPERIMENTAL PROCEDURES

All substances, if not stated otherwise, were obtained from Merck,
Darmstadt, Germany. Avidin-alkaline phosphatase and avidin-peroxi-
dase conjugates, acrylamide, bisacrylamide, arginine, 5-bromo-4-
chloro-3-indolyl phosphate, BSA, DTT, GSH, glycine, iodoacet-
amide, sodium borohydride, N-(N-acetylcysteine (NAC), nitro blue
tetrazolium, SDS, SDS 7B molecular weight marker kit, Tris base,
TWEEN 20, and urea were purchased from Sigma Chemie GmbH,
Deisenhofen, FRG. Lysine was from Serva, Heidelberg, FRG. C1q-de-
ficient serum was from Quidel (San Diego, CA), and C4-deficient
guinea pig serum was provided by the laboratory animal facility of our
institute.

Human Immunoglobulins and Fab′ Fragments—Human (hu) IgG
subclasses 1–4 and polyclonal huIgG were obtained from Sigma. Fab′
fragments of huIgG were purchased from Dianova, Hamburg, FRG,
diluted in PBS, and preadsorbed onto protein G-Sepharose beads
(Pierce, KMF Laborhochards Handels GmbH, St. Augustin, FRG) to
remove any contaminating Fc fragments or complete IgG molecules.
The purity was checked by SDS-PAGE and subsequent Coomassie Blue or
copper staining (21) or by ELISA as described later in this paper.

Specific Antibodies—The following murine (mur) anti- (α)-huC1q
monoclonal antibodies (mAb) are of the IgG1 subclass and were em-
ployed in the present study: 242G3, 241F11, 244G8, 4A4B11, and
12A5B7. The clones 242G3, 241F11, and 244G8 were produced in our
laboratory (22); 4A4B11 and 12A5B7 were obtained from the American
Type Culture Collection (Rockville, MD). The polyclonal goat (gt)
α-huC1q antibody was also produced in our laboratory, and the pol-
cyonal rabbit (rb) α-huC1q antibody was purchased from Behring AG
(Marburg, FRG). Antibodies were purified from serum, ascites, or cul-
ture supernatants by fast protein liquid chromatography using protein
A or G (Pharmacia Biosystems GmbH, Freiburg, FRG) according to the
supplier’s instructions.

The goat α-huC1q Ab was biotinylated using sulfosuccinimidyl-6-
(biotinamido) hexanoate (SSBH; Serva) and, for some experiments,
preincubated onto immobilized huIgG. Briefly, 11.4 mg of purified anti-
body (6 ml) was dialedyzed against 2 liters of 0.1 M NaHCO3, pH 9,
overnight at 4–6 °C. 1.88 mg of SSBH was dissolved in the protein
solution by vortexing, and the mixture was incubated for 4 h at room
temperature. Excess labeling reagent was removed by dialysis against
a specific 5-liter volume of PBS. To reduce background staining when de-
tecting C1q bound to IgG in the ELISA system, 1 ml of labeled Ab was
incubated twice for 1 h at 4–6 °C with a 100-μl packed volume of huIgG
coupled to CNBr-activated Sepharose 4B (0.5 mg IgG/ml beads; IgG-
4B, see below).

Rabbit α-huIgG Ab, goat α-hIgG Ab, and rabbit α-huIgG Ab, all
conjugated to horseradish peroxidase, were obtained from Bio-Rad Mu-
nich (FRG), and a goat α-huC1q Ab coupled to IgG, from Sigma.

Preparation and Labeling of Human C1q—Purification and labeling
(14C)-methylene (23), biotinylation) of human serum C1q were car-
ried out as reported previously for guinea pig serum C1q (24) with the
following additional step in the following C1q purification procedure. Purified
C1q was applied to a protein G column (Pharmacia) equilibrated with the
same protein storage buffer, to remove contaminating IgG as far as possible.
The purity of the C1q preparations was assessed by SDS-PAGE and
ELISA (see below), and only IgG-free preparations were used in exper-
iments in which binding to IgG was investigated.

ELISA Systems—ELISA systems were employed to detect and quan-
tify C1q attached to IgG and also to evaluate the effects on the antige-
nicity of C1q and IgG of the reagents that were used to detach them.

Assay for Assesment of C1q Binding to IgG—96-well microtiter
plates (Maxisorb, Nunc, Wiesbaden, FRG) were coated with 30 μl/well
of a solution in PBS (0.01 M sodium phosphate, 0.145 M NaCl, pH 7.4;
conductivity, 15 mS) containing 250 μg/ml, in some experiments 125 or
62.5 μg/ml, of huIgG or IgG subclasses for 2 h at room temperature or
overnight at 4 °C. As controls, wells were coated with BSA or, in some
experiments with Fab′ fragments instead of whole IgG molecules.

The plates were then washed three times with 200 μl/well PBS
containing 0.3% Tween 20 (PBST) and 1 μl NaCl (conductivity, 63 mS)
followed by three washes with PBST of physiological ionic strength
(conductivity, 15 mS) if not stated otherwise. To block potentially re-
maining binding sites, 100 μl/well of a solution of 1% BSA in PBST was
applied twice for 30 min. After two more washes with PBST, or, in some
experiments PBS containing 0.15 mM Ca2+ and 1 mM Mg2+ (PBS0.15M),
serial dilutions of C1q (concentrations ranging from 0.078 to 20.0 μg/ml)
were applied and incubated for a certain time interval (usually 1 h)
at room temperature or 37 °C, as indicated. Serial Dilution of C1q applied
to wells coated with goat α-huC1q-IgG (1:400 dilution of a stock solution
containing 1.9 mg/ml Ab) instead of huIgG served as quantitative
standards. Controls in which C1q was omitted or incubated in BSA-
coated wells were included in each assay, and all samples were ana-
lized in duplicate. Respective samples were subjected in parallel
to three washes or treatment for a distinct time period with PBST and/or
any other elution buffer as detailed in the text. The plates were
washed sequentially three times each with 20 μl washing buffer in PBST alone.
When BSA-containing C1q standards were always treated only with PBST.
Detection of bound/remaining C1q was accomplished by sequential incubations with biotinylated goat α-huC1q-IgG (1:800) and avidin-peroxidase conjugate (1:750); or, in the case of bioti-
nylated C1q, with avidin-peroxidase conjugate directly; or with a mu-
rine α-huC1q mAb (usually 242G3, 1:400) and a goat α-murIgG horse-
radish peroxidase (1:1,000). All dilutions of Ab or avidin-peroxidase
conjugate were prepared with 20 μl/well PBS A. 2 μl/mg in 10 mM
citrate, pH 4.5, containing 0.01% H2O2 served as substrate; the staining
reaction was terminated by the addition of 100 μl/well PBS. The ab-
sorbance at 405 nm was measured in an Anths Labtech microplate
reader (Anths Labtech Instruments, Salzburg, Austria), and the data
analysis was performed using Microtest software version 4.0,
(Microtest Labosystems, Overath, FRG).

Assessment of the Effects of Elution Buffers Employed to Remove C1q
from Immobilized IgG—Wells coated with huIgG and blocked as de-
scribed above, but not incubated with C1q, were treated in the same
manner as if to liberate attached C1q. The remaining immobilized IgG
was then detected with a rabbit α-huIgG Ab conjugated to horseradish
peroxidase as described above for C1q. In some experiments, wells were
coupled with huIgG containing 0.5, 1, or 2% biotinylated IgG as tracer.
Biotinylation of huIgG was performed as described before for the goat
α-huC1q Ab. After treatment of the immobilized IgG with the respective
elution buffers, the remaining IgG was detected using the avidin-per-
oxidase conjugate. Serial dilutions of IgG served as standards.

Assays for Quanification and Evaluation of Alterations in the Anti-
genicity of C1q—Quantification and analysis of changes of the antige-
nicity of C1q were accomplished using a previously described sandwich
ELISA system (25) with minor modifications. In brief, wells were coated
with 30 μl of a 1:400 dilution of goat α-huC1q-IgG (1.9 mg/ml) in PBS
for 1 h at room temperature or overnight at 4 °C. Remaining binding
sites were blocked with BSA, as described before, followed by two
washes with PBS. Serial dilutions of pretreated C1q or normal human
serum were prepared in PBS or PBS0.15M respectively, with or without

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a final concentration of 20 mM EDTA. Dilutions of purified C1q served as standard. In some experiments, wells were coated with C1q directly instead of Ab. Detection of bound C1q was achieved as described before for C1q attached to hulgG, employing murine α-hulgC1q mAb 242G3 or biotinylated goat α-hulgC1q Ab or avidin-alanine phosphatase conjugate in the case of biotinylated C1q.

Measurement of C1q Hemolytic Activity and C4 Activation—The hemolytic activity of C1q was assayed as reported earlier (26). C4 activity was determined according to a method described by Atkinson and colleagues (27) with the following minor modifications. Imidazole replaced Veronal in all buffers, and the assay was scaled down to 100 µl each, a suspension of IgG-coated sheep erythrocytes (1.5 × 10⁹ cells/ml, dilutions of serum sample, and C4-deficient guinea pig serum containing human C2.

Affinity Adsorption—Human IgG was coupled to CNBr-activated Sepharose beads (Pharmacia), according to the supplier’s instructions, at a concentration of 10 mg of protein/ml of beads. 10 µg of [14C]-labeled C1q in PBS was incubated with IgG-S4B (200-µl packed volume) in a total volume of 400 µl. The beads were kept in suspension by end-over-end rotation for 4 h at room temperature. Then the IgG-S4B was washed three times with 500 µl of PBS and sequentially eluted with the following.

RESULTS

Binding of C1q to Immobilized IgG and Probing of the Stability of the Interaction—Serial dilutions of purified C1q in concentrations ranging from 10 to 0.078 µg/ml in PBST of physiological ionic strength (conductivity, 15 mS) were applied to immobilized hulgG in 96-well ELISA plates and incubated for 1 h at room temperature as described under “Experimental Procedures.” Then the wells were washed with incubation buffer, and the amount of bound C1q was determined using the α-hulgC1q mAb 242G3. The percentage of applied C1q that bound to IgG and thus the binding efficiency correlated inversely to the C1q concentration (Fig. 1A), ranging from about 100% with a C1q concentration of 10 µg/ml to about 40% with 0.078 µg/ml C1q. To assess the stability of the C1q attachment to IgG, the washed wells were washed with PBST containing 1 M NaCl (conductivity, 63 mS) instead of incubation buffer before detection of C1q. Surprisingly, about 40–65% of the C1q amount that was bound in buffer of physiological ionic strength remained attached to IgG (Fig. 1B). The addition of 5% (710 mM) ME to the washing buffers further reduced the amount of detectable bound C1q but was insufficient to dissociate C1q from IgG completely (Fig. 1, C and D). An interaction with C1q and formation of the high salt buffer-resistant association were only observed for the IgG subclasses 1 and 3, but not with IgG of the subclasses 2 or 4 (Fig. 2, A–C). In our assay, C1q bound specifically to immobilized hulgG and thereby to the Fc domain, since no binding to immobilized BSA or F(ab)² fragments occurred (Fig. 2, D and E). Furthermore, C1q bound minimally if the immobilized IgG was reduced with 710 mM ME or 20 mM DTT and alkylated using iodoacetamide, before incubation (Fig. 2, F and G). Increasing the NaCl concentration in the elution buffer up to 3 M or prolonged incubation did not result in a further release of tightly bound C1q (Fig. 3, A and B). An extension of the incubation time with the eluent to 30 min diminished the amount of bound C1q only if the reducing agent ME was present. A treatment for 60 min with the same buffer showed no additional effect (Fig. 3C). C1q did not interact with IgG in the presence of high salt buffers (Fig. 4). Similar obser-
vations on the interaction of C1q and IgG were obtained if C1q was detected with other α-huC1q mAbs or the polyclonal goat α-huC1q Ab or if biotinylated C1q was employed (data not shown). Comparable results were also observed if the concentration of immobilized IgG/well was reduced so that the wells were coated with IgG in concentrations of 125 μg/ml or 62.5 μg/ml instead of 250 μg/ml (see Fig. 2, A–C and below; not all data are shown). Measurement of IgG suggested that, under the conditions of our assay, coating wells with 125 or 250 μg/ml IgG resulted in nearly the same amount of immobilized IgG (data not shown).

Additional Attempts to Detach C1q from IgG—To analyze further the nature of the association, the complex of immobilized IgG and C1q was subjected to treatment with PBST at pH 5.5 or PBST containing various agents such as 20 mM DTT instead of ME, arginine, lysine, and urea, sometimes in combination with ME. Alternatively, the associated proteins were incubated in SDSsb at 60 °C for 30 min, in the presence or

![Figure 2](image2.png)

**Fig. 2.** Specificity of C1q binding to immobilized IgG and IgG subclasses. 96-well microtiter plates coated with huIgG subclasses 1, 2, 3, or 4 were incubated with C1q. Bound C1q was detected after washes with incubation buffer using mAb 242G3 (panels A and D–G) or goat α-huC1q Ab (panels B and C); C1q bound to IgG1 and IgG3, respectively, after treatment in parallel with PBST containing 0.145 M NaCl (K15) or 1 M NaCl (K63) or 1 M NaCl and 710 mM ME (K63 ME). Alternatively, C1q was incubated in wells coated with huIgG or BSA (panel D); a control in all assays or F(ab)2 fragments of huIgG (panel E) or reduced huIgG (panels F and G). Reduction of IgG was performed for 1 h in PBST containing 1 M NaCl and 710 mM ME (panel F) or 20 mM DTT (panel G) after the initial coating of the wells and before blocking with BSA. Free thiol groups were subsequently inactivated by two washes and incubation for 1 h with 200 μl/well each time of 20 mM iodoacetamide in PBST. Representative results of one out of three experiments are shown, respectively. K followed by a number indicates conductivity in mS.

![Figure 3](image3.png)

**Fig. 3.** C1q remaining bound to immobilized IgG throughout treatment with buffers containing NaCl in the absence or presence of ME. Immobilized IgG was incubated with C1q in PBST (0.145 M NaCl) for 1 h at room temperature and then treated with PBST containing 0.58, 1, 2, or 3 M NaCl (panel A, one representative experiment out of three). Alternatively, the proteins were subjected to treatment with 1 M NaCl in PBST, without (panel B) or with 710 mM ME (panel C), for 3 min (three washes), 30 min, or 60 min. C1q resisting elution was detected with mAb 242G3 and is expressed as a percentage of the quantity remaining attached under exposure to PBST of physiological ionic strength (0.145 M NaCl, conductivity, 15 mS). Values ± S.E. of five experiments are shown in panels B and C.

![Figure 4](image4.png)

**Fig. 4.** Incubation of C1q and immobilized IgG in buffers of different NaCl content. C1q was incubated with immobilized IgG for 1 h in PBST containing 0.145, 1, 2, or 3 M NaCl. The mAb 242G3 served to detect bound C1q. One representative out of three experiments is shown.
The experiments were performed as outlined under “Experimental Procedures” and in the figure legends. The results are displayed in Figs. 5 and 6 and show that C1q bound to IgG was still detectable after all of the different treatments except that SDSsb containing 710 mM ME.

There were three major considerations in the dissociation of C1q and IgG: the efficiency of the applied reagents in liberating C1q compared with 1 or 3M NaCl; the effects of the reagent on the immobilized IgG; and the properties of C1q, in particular its antigenicity. The experiments were performed as outlined under “Experimental Procedures.” Immobilized IgG treated with PBS for 30 min at 60 °C served as 100% control.

TABLE I
Reagents used to wash or dissociate the complex of immobilized IgG and C1q

| Buffer        | Substance(s) | Concentration | Conductivity | Resid. IgG |
|---------------|--------------|---------------|--------------|------------|
| PBST          | NaCl         | −0.145        | 15           | No effect  |
| PBST          | Arginine     | 0.001; 0.005; 0.01 | 15         | No effect  |
| PBST          | Lysine       | 0.001; 0.005; 0.01 | 15         | No effect  |
| PBST          | Urea         | 0.5           | 15           | 91.1 (±4.1) |
| PBST          | Urea         | 1.0           | 15           | 112.0 (±3.8) |
| PBST          | Urea         | 2.0           | 15           | 97.6 (±1.9)  |
| PBST          | Urea         | 5.0           | 15           | 103.8 (±10.1) |
| SDSsb         | SDS          | (2%)          | ND           | 18.1 (±5.9)  |
| SDSsb         | SDS/ME      | (2%)/0.71     | ND           | 3.0 (±0.7)   |

a NaCl was included in all buffers.

b Conductivity was adjusted with NaCl at room temperature; ND, not determined.

c Microtiter plates were always washed with 1 M NaCl after coating with IgG. Mean percentage of residual, fixed IgG after treatment (±S.E.) of four determinations.

d PBST (0.145 M NaCl) is the binding buffer for C1q (see “Experimental Procedures”).

e For contents, see “Experimental Procedures.” Immobilized IgG with SDSsb and elution buffers containing a reducing agent (ME or DTT) served the eluent and was detected with avidin-peroxidase. One representative experiment out of three is shown.

Potential effects of the elution buffers/reagents on the antigenicity and function of C1q were assessed by two approaches. In one approach C1q was immobilized in wells of a microtiter plate, subsequently treated with the respective buffers, and then detected in the same way as if bound to immobilized IgG (see “Experimental Procedures”). In the other approach soluble C1q (0.3–6.5 μg) was incubated in a total volume of 100–110 μl of PBS for 1 h at room temperature with the respective molar concentration of urea. After dialysis against three 100-ml volumes of PBS, antigenicity and hemolytic activity of C1q were analyzed as outlined under “Experimental Procedures.”

The first approach showed that arginine, lysine, pH 5.5, up to 3 M NaCl and up to 2 M urea had no effect on the antigenic detection of C1q (data not shown). Treatment with 1 M NaCl in the presence of ME (or DTT) reduced the amount of detected C1q as the C1q concentration increased (Fig. 6). Exposure to 5
as described above for C1q bound to immobilized IgG. C1q washed with
soluble C1q was removed), 1 h and 4 h (Fig. 7, PBST after 15 min, and the incubation was continued for up
to the supernatant. Therefore we performed experiments in
increasing strength of the association depended on free C1q in
immobilized IgG. This finding suggested that the interaction of
than formation of the tight attachment of C1q and IgG occurs with time and independently of
free soluble C1q in the supernatant.

**Incubation of C1q with IgG Coupled to Sepharose Beads**—In
an alternative approach to the ELISA system, 14C-labeled C1q
was incubated with IgG-S4B for 4 h at room temperature. Subsequent
treatment with incubation buffer, 0.58 m NaCl, SDSs, and SDSs containing ME was performed as described under “Experimental Procedures” to elute C1q. Analysis of the
euates by SDS-PAGE and fluorography revealed that C1q
could only be liberated under the influence of SDSs. SDSs alone
removed only a part of the bound protein since subse-
quent reduction with ME caused a further release of C1q (Fig.
8). Densitometric estimation showed that SDSs removed
48.1 ± 9.9%, and SDSs containing ME removed 51.9 ± 9.9% of
all eluted C1q (mean ± S.D. of three experiments). The ob-
erved binding of C1q was specific for IgG since no adsorption
onto Sepharose-coupled BSA was detected (data not shown, but
see Ref. 29).

**Incubation of Serum with Immobilized IgG**—Because C1q in
serum usually acts in concert with C1r and C1s and thus exerts
its function as a part of the C1 complex, we wondered whether
the tight binding of C1q to IgG could also occur in serum. Serial
dilutions of a pooled serum containing 250 µg C1q/ml in PB-
SCa, Mg were applied to 96-well plates coated with IgG and
incubated for 1 h at 37 °C. Then the serum dilutions were
removed, and IgG-bound C1q was immunologically assessed after washes with PBST or PBST containing 1 m NaCl (± ME) as
described before. Additionally, the residual C4 activity in the
incubated serum dilutions was determined as a measure of
classical complement pathway activation. Serum dilutions ap-
plied to wells coated with BSA or containing 20 mM EDTA and
purified serum C1q served as controls. The result, shown in
Fig. 9A, revealed that tight binding of C1q to IgG developed in
serum in the presence and absence of EDTA. Purified C1q and
C1q in serum containing EDTA behaved similar to each other
with respect to binding to IgG but were distinct from C1q
engaged in the C1 complex in normal serum. Binding of C1r-
and C1s-associated C1q (C1) was less effective at higher, but
more efficient at lower concentrations, compared with free C1q
in serum containing EDTA. Activation of serum C4 occurred
only throughout incubation with immobilized IgG and in the
absence of EDTA. Furthermore, C4 consumption correlated
well with the observed binding of C1q to IgG.

**Incubation of a Treated, Immobilized C1q-IgG Complex with
C1q-deficient Serum**—To investigate if the tightly IgG-bound
C1q is capable of activating the classical pathway of comple-
ment, the following experiment was performed. IgG-associated
C1q was sequentially treated with PBSCa, Mg containing 0.145
or 1 m NaCl or 1 m NaCl containing 710 mM ME and 20 mM
iodoacetamide in PBSCa, Mg as outlined under “Experimental Procedures.” After equilibration by three washes with
PBSCa, Mg, C1q-deficient serum was added, and the microtiter
plates were incubated for 1 h at 37 °C. Thereafter, the serum
was removed and assayed for remaining C4 activity as de-
scribed above. C1q attached to IgG was detected immuno-
chemically after three washes with PBST (not shown). C1q-
deficient serum incubated with immobilized IgG alone or
applied to wells coated with BSA and preincubated with or
without C1q served as controls. Compared with the controls,
IgG-attached C1q exerted a remarkable ability to activate C4,
also after treatment with 1 m NaCl and even to some degree
after exposure to 1 m NaCl with 710 mM ME (Fig. 9B).

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**Fig. 6. Immunodetection of C1q after exposure to buffers con-
taining 1 m NaCl or 5 m urea with or without reducing agent.** C1q
adsorbed onto microtiter plates was washed three times with PBST
containing 1 m NaCl or 5 m urea in the presence or absence of 710 mM ME
as described above for C1q bound to immobilized IgG. C1q washed with
PBST served as standard, and the goat α-huC1q Ab was used for
detection.

M urea diminished the detection signal obtained with the poly-
clonal goat α-huC1q Ab, the effect being more pronounced if
ME was present (Fig. 6), and it completely abolished recogni-
tion of C1q by the α-huC1q Ab MAb 242G3 (data not shown). After
treatment with SDSs at 60 °C C1q could still be detected with the
goat α-huC1q Ab. If SDSs containing ME was applied, the
detected C1q amount was reduced to about 12% of that as-
seeded after treatment with SDSs alone. Exactly the same
effect of SDSs ± ME was observed with biotinylated C1q (data
not shown).

The second approach confirmed that treatment with 0.5—2 m
urea did not change the antigenicity of C1q. Furthermore, the
hemolytic activity of C1q was recovered after removal of urea
d by dialysis (data not shown). In contrast, pretreatment with 5 m
urea destroyed completely and irreversibly the hemolytic
function of C1q as well as its capability of binding to Fc of IgG
and depressed the antigenic recognition in the C1q sandwich
ELISA to about 25%, compared on the basis of equal protein
amounts. (The biotinylated goat α-huC1q Ab had to be used for
detection since C1q treated with 5 m urea retained no reactivity
with MAb 242G3; data not shown.)

**Kinetics of C1q Binding to IgG and Formation of the High
Salt-resistant Association**—Unlabeled or biotinylated C1q was
incubated with immobilized IgG for different time periods and
subsequently washed with PBST containing 0.145 m NaCl (in-
cubation buffer) or 1 m NaCl with or without ME. Bound C1q
was detected immunochromically or with avidin-alkaline phosphatase conjugate, respectively, as described under “Experi-
mental Procedures.” The results (Fig. 7, A and B) showed that
the amount of C1q remaining elution with 1 m NaCl increased
with time. After 4 h, more than 80% of the bound C1q remained
associated with IgG throughout exposure to 1 m NaCl. Al-
though the detected amount of bound C1q was always lower
after treatment with buffers containing 710 mM ME, the quan-
tity of C1q withstanding the dissociating effect of the reducing
agent increased simultaneously (data not shown).

**Binding of soluble C1q to immobilized IgG occurred faster
than formation of the tight association between bound C1q and
immobilized IgG.** This finding suggested that the interaction of
C1q and IgG proceeds in two steps, and we wondered if the
increasing strength of the association depended on free C1q in
the supernatant. Therefore we performed experiments in
which the supernatant containing soluble C1q was replaced by
PBST after 15 min, and the incubation was continued for up
to 4 h. Bound C1q was determined after 15 min (at the time when
soluble C1q was removed), 1 h and 4 h (Fig. 7, C and D). The
amount of associated C1q barely changed over time in the
absence of soluble C1q, but the quantity of C1q resisting elu-
tion with 1 m NaCl (± ME) increased remarkably, as in the
presence of soluble C1q. Thus, formation of the tight attach-
ment of C1q and IgG occurs with time and independently of
free soluble C1q in the supernatant.
Approaches to Influence the Formation of the Highly Stable C1q-IgG Complex—To analyze the formation of the tight association of C1q and IgG the potential influence of Ca\(^{2+}\), Mg\(^{2+}\), EDTA, temperature, pH, a reducing agent (NAC) and the thiol-blocking substance iodoacetamide was investigated.

The addition of Ca\(^{2+}\) and Mg\(^{2+}\) ions (0.15 and 1 mM, respectively) or of their chelator EDTA (20 mM) showed no effect on the tight binding of C1q to IgG (data not shown).

Performance of the binding assays at 4, 22, and 37 °C revealed that binding of C1q to IgG was achieved at all temperatures. Binding was most pronounced at 22 °C, slightly less effective at 4 °C, and to the lowest extent at 37 °C (Fig. 10A). But formation of the high salt-resistant association occurred markedly only at 22 and 37 °C (Fig. 10B). The reduced binding of C1q at 37 °C compared with 22 °C might be caused by a slight increase of conductivity from 15 to 17 mS at a constant salt concentration. However, the tight attachment of C1q to IgG is influenced by the temperature and strongly impaired in the cold.

If C1q and immobilized IgG were incubated in PBST of different pH, ranging from 5.5 to 8.5, the observed binding was the best at pH 7.4 (Fig. 10C). At least for C1q concentrations below 2.5 mg/ml the attachment was inhibited at any pH higher than 7.4. Impairment of the C1q-IgG interaction was even more pronounced in buffers of any pH below 7.4. The development of the tight, 1M NaCl-resistant association was also strongly diminished at pH below 7.4 but appeared to be not changed at pH 8.0 and even increased slightly for C1q concentrations below 2.5 mg/ml at pH 8.5 (Fig. 10D).

The association of C1q and IgG depends strongly on intact disulfide bridges of both proteins since prior reduction of one of both is sufficient to prevent the interaction (see Fig. 2 and Ref. 30). To assess a potential role of disulfides in the formation of the tight attachment, we sought a reducing agent that would not abrogate the interaction but allow us to regulate it. Out of four substances considered in pilot experiments, ME, DTT, glutathione, and NAC, the last one appeared to be most suitable. Immobilized IgG was treated with 0.2, 2, or 20 mM NAC in PBST followed by iodoacetamide for 1 h before incubation with C1q. No difference in C1q attachment was observed after incubation of C1q with immobilized IgG for 15 min, the C1q dilution was replaced by pure buffer. Bound C1q (panel C) and the percentage of it which resisted elution with 1 M NaCl buffer (panel D) were determined using the goat α-huC1q Ab at the time of the buffer exchange and after 60 and 240 min. One representative out of three experiments is shown.
amount revealed a dose-dependent reduction not only of binding in 0.145 M NaCl but also an impaired formation of the 1 M NaCl-resistant association (Fig. 11, B and C). About 30% of the bound C1q resisted elution with 1 M NaCl after incubation without NAC, but only about 20% and about 15% and less, depending on the C1q concentration applied, after incubation in the presence of 2 and 20 mM NAC, respectively. NAC did not inhibit the antigenic detection of C1q (data not shown).

To address the question of whether free and accessible sulfhydryl groups are involved in the C1q-IgG interaction, we performed C1q binding assays in the presence of 0.2, 2, or 20 mM iodoacetamide. However, neither the C1q-IgG interaction nor the antigenic detection of C1q was affected by the thiol-blocking reagent (data not shown). Furthermore, iodoacetamide did not influence the hemolytic activity of C1q if present in concentrations ranging from 10 to 250 mM during the incubation of C1q with IgG-coated erythrocytes (data not shown).

**Ligand Blotting—C1q consists of three similar but distinguishable protein chains. To analyze if all of these can contribute to the interaction with IgG, the A, B, and C chains were**

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**Fig. 9. C1q binding and C4 activation. Panel A, serial dilutions of a human serum pool in PBS(A-M) were incubated in the presence or absence of 20 mM EDTA with immobilized IgG (125 µg/ml used for coating of wells) or, as a control, with immobilized BSA, for 1 h at 37 °C in microtiter plates. Then the serum dilutions were removed, and the residual C4 activity was determined as described under “Experimental Procedures.” Samples incubated with BSA or containing EDTA served both as negative controls. C4 consumption in serum samples incubated with IgG in the absence of EDTA is shown and indicated on the right side of the graph. The microtiter plates were treated as described for the C1q binding assay with PBST or PBST and 1 M NaCl, and bound C1q was subsequently detected using the mAb 242G3. Panel B, after incubation of C1q (5 µg/ml) with immobilized IgG for 1 h in a microtiter plate, the wells were washed with PBS or PBS containing 1 M NaCl without or with 710 mM ME as described before. Subsequent incubation with C1q-deficient serum and assessment of C4 consumption were performed as described (27). The mean percentage ± S.D. of C4 consumption in C1q-deficient serum observed in three experiments is shown for the incubation with C1q bound after washes with PBS (K15), PBS and 1 M NaCl (K63), and K63 with ME (K63ME), where K indicates conductivity in mS.**

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**Fig. 10. Influence of temperature and pH on C1q binding to immobilized IgG and formation of the tight, high salt buffer-resistant association in 0.145 M NaCl buffer. The C1q binding assay was performed in microtiter plates as described under “Experimental Procedures” but at 4 °C, 22 °C (room temperature), and 37 °C. IgG-bound C1q was detected employing the mAb 242G3 after washes with PBST (panel A, mean absorbancies ± S.D. obtained in three experiments) or PBST containing 1 M NaCl (panel B, percentage of C1q bound in PBST). The C1q binding assay was performed in PBST of the indicated pH. Total bound (panel C) and 1 M NaCl treatment-resistant attached C1q (panel D) were assessed employing the mAb 242G3. The results are displayed as percentages in relation to C1q which remained attached after the same elution procedure after incubation at pH 7.4. One representative out of three experiments is shown.**
separated by SDS-PAGE under reducing conditions and transferred onto a polyvinylidene difluoride membrane. Subsequent to saturation of the blotting membrane, the separated C1q chains were incubated with huIgG or IgG subclasses as detailed under “Experimental Procedures.” The ligand blotting revealed that all three chains were able to bind IgG in the presence of 70 or 145 mM NaCl (Fig. 12). Binding to the B chain could hardly be detected after incubation with IgG at a concentration of 10 μg/ml but clearly after applying 50 μg/ml. Interestingly, the separated C1q chains bound IgG1 and 3 but not IgG4. IgG attachment to C1q was specific since no binding to BSA occurred. Furthermore, 1M NaCl or 710 mM ME failed to remove bound IgG from the C1q chains. In contrast, no IgG remained attached through the washes with SDSsb.

**DISCUSSION**

The present study addresses two currently open questions concerning the understanding of the interaction of complement with immunoglobulins: how constant the C1q-IgG association is and how the three protein chains of C1q contribute to this association.

The binding of C1q to immobilized IgG was highly specific under the conditions of our ELISA system since, first, C1q bound selectively to IgG subclasses 1 and 3; second, no association with F(ab)92 fragments was observed, which indicates that the interaction is mediated by the Fc region; third, C1q barely attached to reduced IgG; and finally, no binding to immobilized BSA occurred (Fig. 2). However, the percentage of applied C1q that bound to IgG, which can be taken as a measure of the binding efficiency, varied among different C1q preparations but was always correlated inversely with the C1q concentration (Fig. 1).

Direct fixation of IgG in the wells was preferred over antigen-bound antibody because we intended to expose the complex of C1q and immobilized IgG to reagents that would probably also interfere with the antigen-antibody interaction. However, some of the buffers employed to detach C1q from immobilized IgG, such as SDSsb or buffers containing reducing agents, removed also certain amounts of immobilized IgG (Table I). Because eluted IgG might have carried away C1q, an underestimation of the C1q quantity remaining attached under these conditions is likely.

To investigate how constant the interaction is, we treated the complex of C1q and immobilized IgG with buffers that were slightly acidic (pH 5.5) or buffers containing arginine, lysine, or high concentrations of salt or denaturing substances, the last two in the presence or absence of reducing agents, respectively (Table I). Subsequent to each of the various elution procedures, which left behind at least about 12% of the immobilized IgG, C1q binding to untreated IgG in the presence of 1–3 M NaCl (Fig. 3) or at pH 5.5 (Fig. 10). Furthermore, 5 M
urea abrogated irreversibly the IgG binding ability of C1q (data not shown, but see Ref. 30). But once C1q was attached to IgG the respective agents or conditions failed to reverse the binding completely. Furthermore, the reagents that eluted a certain amount of C1q but which did not affect C1q antigenicity (arginine, 1–3 M NaCl, or 1 or 2 M urea) appeared to be equally effective in releasing C1q (Fig. 5). Altogether, our results indicate for the first time that under certain conditions the binding of C1q to IgG is not reversible, in contrast to earlier suggestions by others (32). A part of C1q remained tightly attached to IgG even during exposure to usually strongly dissociating or denaturing agents such as 1 or 3 M NaCl, up to 5 M urea or SDS and heating, indicating that a change in the quality of the C1q-IgG interaction had occurred.

C1q that had been incubated in suspension with IgG coupled to Sepharose beads also resisted elution with high salt buffer and SDSsb, indicating that the tight association could be a general feature of the C1q-IgG interaction. A closer investigation revealed that the binding of soluble C1q to immobilized IgG occurred faster than the formation of the high salt-resistant association between both proteins. The strengthening of the interaction of bound C1q and IgG was found to be time-, temperature- and pH-dependent, even in the absence of free C1q in the supernatant (Figs. 7 and 10). This demonstrated first that the attachment of C1q to IgG proceeds in two steps; second, that two qualitatively distinct states exist in the association of C1q and IgG; and third, that the C1q-IgG interaction cannot only be described in terms of an equilibrium process as suggested earlier (32). The first stage of the C1q-IgG association is characterized by its reversibility in the presence of, for example, high salt concentrations, and the following second stage by its constant nature under conditions that usually dissociate the protein-protein interaction, such as the presence of high salt buffer or denaturing agents.

Based on our results and the following facts, we suppose that the strengthening of the C1q-IgG association is a consequence of a time-dependent arrangement of β-sheet interactions between both proteins. First, the globular regions of C1q appear to be to a large extent folded as β-sheets (4). Second, IgG domains form β-sheets. Third, the binding site for C1q on the Cy2 domain is located on the fý2 β-sheet (32). Fourth, C1q enhances β-structure formation in amyloid β-peptide aggregates (33). Finally, aggregates consisting of β-structure are highly resistant to solubilization or dissociation (33).

Interesting questions are whether the two stages of the C1q-IgG association are reflected by changes in the antigenicity of C1q and IgG and whether the stages display functional differences. In the present study, we did not assay for antigenic changes in IgG. We preferred to use unlabeled C1q and its antigenic detection to keep the manipulation of the protein to a minimum. Furthermore, a targeted labeling of C1q in whole serum would have been impossible. However, the results we obtained using a mAb or a polyclonal Ab to detect IgG-bound C1q did not indicate the appearance of neoantigenicity throughout the formation of the high salt buffer-resistant C1q-IgG complex, but neither do they permit exclusion of it. Further investigations employing a reasonable, but not yet available set of mAbs may be required to address this point in more detail.

Some of the elution buffers influenced the immunodetection of C1q per se, in particular 5 M urea, SDSsb, and the buffers including a reducing agent. Therefore, the reduction of the amount of C1q observed after exposure to one of these buffers could be caused by detachment from immobilized IgG or destruction of the C1q structure or by impaired reactivity from disappearance or alteration of epitopes. To overcome at least the disadvantage of epitope loss or change, we employed biotinylated or 14C-labeled C1q in some experiments. The biotin residue and the radioactive carbon are both linked covalently to the C1q molecule, and their detection is therefore independent of the antigenicity of the protein.

Tight binding of C1q to IgG occurred in serum among C4 activation, which lends additional support to the assumption that it is a physiological event. But C1q engaged in the C1 complex and free C1q released from C1 in the presence of EDTA differed in their behavior (Fig. 9A). C1r2/C1s2-associated C1q interacted most efficiently with IgG at lower concentrations compared with its free counterpart. This indicated that C1r and C1s not only mediate the activation of C4 by C1 but also influence the C1q-IgG interaction and therefore probably the recognition of IC by the classical pathway of complement. Tightly attached C1q was readily capable of mediating C4 activation after exposure to the elution buffers (Fig. 9B). However, our results did not permit identification or exclusion of difference(s) between the amounts of reversibly and irreversibly IgG-bound C1q in terms of a biological function such as the extent of C4 activation. The strengthening of the C1q-IgG interaction could possibly prolong the availability of the C1q-IgG complex for initiation of the classical pathway of complement without changing the quality or extent of the activation event itself.

Biotinylation and 14C-labeling both involve preferentially free amino groups provided by lysine residues. But we and others have observed that they do not interfere with the IgG binding capability or the hemolytic activity of C1q (23, 34). The behavior of biotinylated and unlabeled C1q toward immobilized IgG was identical in our binding assays. In contrast, biotinylation of IgG has been shown to abrogate the binding of C1q and activation of the classical pathway of complement (35).

Therefore, it appears that (free and accessible) lysine residues of IgG but not of C1q play a role in the interaction of both proteins. Sufficient association of C1q and IgG depends strongly on intact disulfide bridges in both proteins since prior reduction of one of the molecules prevents their association (Fig. 2 and Refs.
30 and 36). Our observation that the reducing agent NAC led to a dose-dependent reduction not only of C1q binding to IgG in 0.145 M NaCl but also to an impaired formation of the 1 M NaCl-resistant association (Fig. 11) indicated that disulfide bridges play a prominent role in the strengthening of the C1q-IgG association. This view is supported by the finding that at pH 8.5 compared with pH 7.4 the relative amount of C1q resisting high salt buffer elution increases (Fig. 10), although the binding of C1q to IgG is in general impaired. Disulfide interchange reactions are favored at pH 8.5 (37), and C1q has been observed to form disulfide-linked oligomers under these conditions (38). Interestingly, a half-cystine is located within the binding site for C1q on IgG in position 321 between Lys-320 and Lys-322 on the α2 β-sheet (see Fig. 2 in Ref. 32). Although we do not have any direct experimental evidence, one could speculate that this half-cystine of IgG could be involved in a disulfide interchange reaction with a matching counterpart in C1q. The results of our experiments would be consistent with this hypothesis. However, free and accessible sulfhydryls are probably not required for the C1q-IgG interaction, since iodoacetamide, which blocks thiol groups and has the potential to interfere with disulfide interchange (37), did not influence the C1q-IgG interaction. But C1q possesses cryptic sulfhydryls (30, 39), and therefore, disulfide interchange reactions could occur in a concealed molecular microenvironment, inaccessible to iodoacetamide.

Our ligand blotting experiments demonstrated for the first time that each of the three C1q protein chains contributes to the interaction with IgG, and thus we have extended the findings of others that a recombinant fragment of the B chain globular region (17) and the C chain (16) are both capable of bindings of others that a recombinant fragment of the B chain. Additionally, our study shows for the first time that separated C1q chains bind selectively to IgG sub-

**REFERENCES**

1. Reid, K. B. M. (1979) *Bioch. J.* 179, 367–371
2. Knobel, H. R., Villiger, W., and Isliker, H. (1975) *Eur. J. Immunol.* 5, 78–81
3. Reid, K. B. M., and Porter, R. B. (1976) *Bioch. J.* 155, 19–23
4. Smith, K. F., Harris, P. I., Chapman, D., Reid, K. B., and Perkins, S. J. (1994) *Biochem. J.* 301, 249–256
5. Marques, G., Anton, L. C., Barrie, E., Sanchez, A., Ruiz, S., Gavilanes, F., and Vivanco, F. (1993) *J. Biol. Chem.* 268, 10393–10402
6. Tschopp, J., Villiger, W., Lustig, A., Jaton, J. C., and Engel, J. (1980) *Eur. J. Immunol.* 10, 529–535
7. Poon, P. H., Phillips, M. L., and Schumaker, V. N. (1985) *J. Biol. Chem.* 260, 9357–9365
8. Hughes-Jones, N. C., and Gardner, B. (1979) *Mol. Immunol.* 16, 697–701
9. Painter, R. H. (1993) *Behring Inst. Mitt.* 93, 131–137
10. Loos, M. (1983) *Curr. Top. Microbiol. Immunol.* 102, 1–56
11. Bindon, C. I., Hale, G., Bruggemann, M., and Waldmann, H. (1988) *Exp. Med.* 168, 127–142
12. Duncan, A. R., and Winter, G. (1988) *Nature* 332, 738–740
13. Easterbrook Smith, S. B. (1983) *Bioc. Rep.* 3, 135–140
14. Takada, A., Shirahama, S., and Takada, Y. (1984) *Immunopharmacology* 8, 27–35
15. Wines, B. D., and Easterbrook Smith, S. B. (1990) *Mol. Immunol.* 27, 221–226
16. Jiang, H., Robey, F. A., and Gewurz, H. (1992) *J. Exp. Med.* 175, 1373–1379
17. Kishore, U., Leigh, L. E. A., Willis, A. C., Eggleton, P., and Reid, K. B. M. (1996) *Mol. Immunol.* 33, 88 (abstr.)
18. Xu, Y., Oomen, R., and Klein, M. H. (1994) *J. Biol. Chem.* 269, 3469–3474
19. Antes, U., Heinz, H. P., and Loos, M. (1988) *Arthritis Rheum.* 31, 457–464
20. Fukushima, Y., Ouyama, K., Komuro, K., Ueda, M., Fukutake, K., and Fujimaki, M. (1991) *Rinsho. Ketsueki.* 32, 1540–1546
21. Lee, C., Levin, A., and Branton, D. (1987) *Anal. Biochem.* 166, 308–312
22. Golan, M. D., Burger, D., and Loos, M. (1982) *J. Immunol.* 129, 445–447
23. Tenner, A., Lesavre, P. H., and Cooper, N. R. (1981) *J. Immunol.* 126, 2106–2174
24. Antes, U., Heinz, H. P., and Loos, M. (1994) *J. Immunol. Methods* 74, 299–306
25. Golan, M. D., Hitschfeld, T., and Loos, M. (1981) *FEBS Lett.* 128, 281–285
26. Atkinson, J. P., McGinnis, K., and Shreffler, D. (1980) *J. Immunol. Methods* 33, 351–368
27. Laemmli, U. K. (1970) *Nature* 227, 680–685
28. Kaul, M., and Loos, M. (1990) *J. Immunol.* 145, 5795–5802
29. Heusser, C. H., Boesman, M., Knobel, H. R., Jacot Guillarmod, H., and Isliker, H. (1975) *Immunochemistry* 12, 213–219
30. Takada, A., and Takada, Y. (1988) *Immunology* 67, 679–685
31. Burton, D. R., Boyd, J., Brampton, A. D., Easterbrook-Smith, S. B., Emanuel, E. J., Novotny, J., Radmacher, T. W., Schravendijk, M. R., Sternberg, M. J. E., and Dwek, R. A. (1980) *Nature* 288, 338–344
32. Webster, S., O’barr, S., and Rogers, J. (1994) *J. Neurosci. Res.* 39, 448–456
33. Storm, D., Loos, M., and Kaul, M. (1996) *J. Immunol. Methods* 199, 87–99
34. Jokiranta, T. S., and Meri, S. (1993) *J. Immunol. Methods* 151, 2124–2131
35. Isenman, D. E., Dorrington, K. J., and Painter, R. H. (1975) *J. Immunol.* 114, 1726–1729
36. Jansen, E. V. (1959) *Science* 130, 1319–1323
37. Martin, H., Heinz, H. P., Reske, K., and Loos, M. (1987) *J. Immunol.* 138, 3860–3867
38. Martin, H., Kaul, M., and Loos, M. (1990) *Eur. J. Immunol.* 20, 1641–1645

**Acknowledgments**—We thank Elke Doerr and Sabine Pauls for excellent technical assistance and Dr. Andrew Ullmann for critically reading the manuscript.