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Crystallization of an Intact Monoclonal Antibody (4B7) Against *Plasmodium falciparum* Malaria with Peptides from the Pfs25 Protein Antigen

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Abstract

Monoclonal antibody 4B7 is a neutralizing antibody that binds the protein Pfs25 in the sexual stages of the malaria parasite *Plasmodium falciparum* and completely blocks transmission of the parasite from human serum to the mosquito host. Here we report the identification of the epitope on Pfs25 recognized by 4B7 and the crystallization of the intact murine monoclonal antibody with peptides corresponding to that epitope. This study highlights the importance of ligands in the crystallization of proteins. In this case peptides have been used to modulate the solubility of the peptide-IgG complex and may have provided different or additional crystal contacts to create or enhance a crystalline reticulum. Multiple crystal forms characterize this crystallization and the various peptides, differing both in length and sequence, have been used to investigate how such changes affect nucleation and crystal growth.

Introduction

Pfs25 is a protein expressed on the surface of zygote, gamete and ookinete forms of the malaria parasite, *Plasmodium falciparum*, as they develop in the midgut of the mosquito (Vermeulen *et al.*, 1985). The Pfs25 antigen has been identified as a major target in blocking the transmission of malaria (Kaslow *et al.*, 1988) and is under consideration as a component for a multistage vaccine aimed at reducing the incidence of the disease. Its inclusion in a vaccine could prevent the transmission of escape mutants and avoid the selection of a vaccine-resistant parasite population. For this reason, the development and understanding of the mode of action of neutralizing antibodies against ookinetes is of interest. Neutralizing monoclonal antibodies such as MAb 4B7 (Barr *et al.*, 1991) are of interest for vaccine development and efforts are underway to determine the structure of MAb 4B7 and its complexes with linear and cyclic peptides (Stura, Kang *et al.*, 1994). The linear epitope recognized by this MAb is closely related to those previously mapped for other transmission-blocking MAbs, 32F81 and 32F61 (Van Amerongen, Sauerwein, Beckers, Meloen & Meuwissen, 1989). The significance of this epitope as part of a transmission-blocking target site is further supported by the capacity of peptides from this site to compete with parasite-produced Pfs25 for binding to the MAb 32F81 (Van Amerongen *et al.*, 1992). The epitope is contained within the sequence ILDTSNPVKT from the third epidermal growth factor (EGF)-like domain of the Pfs25 molecule.

Crystallization of the intact immunoglobulin was attempted after papain and pepsin trials failed to produce an Fab or $F(ab')_2$ which could be later reduced to give an Fab', but resulted in degraded material or uncleaved IgG. Attempts at crystallization of intact IgG have been discouraged by the disordered Fc in the KOL structure (Colman, Deisenhofer, Huber & Palm, 1976; Marquart, Diesenhofer, Huber & Palm, 1980) and the inherent conformational heterogeneity conferred on the immunoglobulin by the flexibility of the hinge. Since 1977 (Silverton, Navia & Davis, 1977) the only intact immunoglobulin structure known had a deleted hinge region. This remains the highest resolution structure determined (Rajan *et al.*, 1983). The crystallization of an intact immunoglobulin by Larson, Day, Greenwood, Skaletsky & McPherson (1991) and the determination of the arrangement of their domains (Harris *et al.*, 1992), has been an important breakthrough in the field. With advances in purification technology and crystallization methodology, as well as in molecular-replacement programs (see review by Wilson, Rini, Fremont, Fieser & Stura, 1991), IgG crystallization and structure determina-

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tion, which bypasses the need to determine and optimize cleavage conditions to produce Fab's, may eventually become a valid alternative to Fab fragments for structure determination of antigen-antibody complexes. However, further advances in methodology are still needed before this alternative will yield structures of comparable resolution to those that can be currently achieved from Fab fragments.

The work described here is directed towards understanding crystallization of IgG, in particular in complex with antigens. Here we report the crystallization of IgG 4B7-peptide complexes. Crystals of the Fab fragment of 4B7 (obtained by elastase cleavage; Stura, Kang *et al.*, 1994) have also been obtained and will be used to obtain a high-resolution structure (2.3-3.2 Å) of the antigen-antibody complex. Crystals of the intact IgG diffract to only 5 Å, suitable for the determination of the domain arrangement of the Fab and Fc. This information could be combined with the high resolution of the Fab-peptide complex to build a complete structure for the intact IgG-peptide complex. While this remains an option, the long-term goal is to obtain better diffracting crystals of the intact IgG complexed to a peptide or to the entire Pfs25 antigen. Although some information about the distribution of domains in intact immunoglobulins can be deduced from electron microscopy (Wrigley, Brown & Skehel, 1983; Roux, 1985; Lamy *et al.*, 1985) and other methods, crystallographic studies are capable of producing additional detail to further our understanding of complement activation and the interactions with the Fc receptor (Wade, Taveau & Lamy, 1985; Dangl *et al.*, 1989; Schneider, Wensel, Stryer & Oi, 1988; Van Haeringen, Jiskoot, Van Grondelle & Bloemendal, 1992; Zheng, Shopes, Holowka & Baird, 1992; Tan, Shopes, Oi & Morrison, 1990; Hoekzema, Martens, Brouwer & Hack, 1988; Duncan, Woof, Partridge, Burton & Winter, 1988; Duncan & Winter, 1988). These studies will add structural information to the discussion of the influence of antigen binding on IgG structure and of the hinge-region tether on the mechanism by which C1q or Fc-receptor binding is achieved. These crystallization studies are aimed towards building the foundations for further work for the crystallizations of IgC and its complexes with complement molecules and Fc receptors.

Materials and methods

Epitope mapping by PEPSCAN

Overlapping nonapeptides were synthesized on solid phase (Van Amerongen *et al.*, 1989) on the basis of the previously reported amino-acid sequence for Pfs25 (Kaslow *et al.*, 1988) This synthesis and subsequent immunoscreening with an ELISA-type analysis (PEPSCAN) was carried out according to established procedures (Geysen, Meloen & Barteling, 1984). The same net of nonapeptides used for mapping the antigenic site of MAb 32F81 (Van Amerongen *et al.*, 1989) was used for MAb 4B7 (Barr *et al.*, 1991) (Fig. 1).

Preparation and purification of 4B7 IgG

The first stage of the preparation consisted of a 20% saturated ammonium sulfate precipitation to separate out the IgG from the less soluble components of the ascites. The IgG was further purified from the resultant supernatant by precipitation with 50% saturated ammonium sulfate. The sample was then centrifuged at 10 000 rev min^{-1} at 277 K for 40 min. The pellet containing the IgG was resuspended in 0.1 *M* sodium acetate, 0.02% NaN_3, pH 5.5 and dialyzed against two changes of the same buffer, with 12 h between changes. The resulting IgG was analyzed by SDS-PAGE on a Pharmacia Phast System and confirmed to have an apparent molecular weight (M_r) of 150 kDa. The IgG was separated over a Sephacryl S-300 size-exclusion column, with a running buffer consisting of 0.1 *M* sodium acetate, 0.2 *M* NaCl, 0. 02% NaN_3, pH 5.5. The purified IgG was concentrated to 5.4 mg ml^{-1}. Further purification of the IgG was carried out using a Mono-S column (Pharmacia) on an HPLC system equilibrated with 0.05 *M* sodium acetate, 0.02% NaN_3, pH 5.5, and eluted with 1 *M* NaCl under the same buffer conditions, The elution profile consisted of a main peak, and a sizable shoulder. Pooled samples from both the peak and shoulder have been analayzed separately in the crystallization studies. Measurements by light diffuse scattering with a Biotage dp801 instrument were consistent with a intact immunoglobulin and showed that the samples was monodisperse.

The IgG isotype determined by two different kits obtained from Zymed and Southern Biotechnology Associates, was a γ2a-heavy chain and κ-light chain.

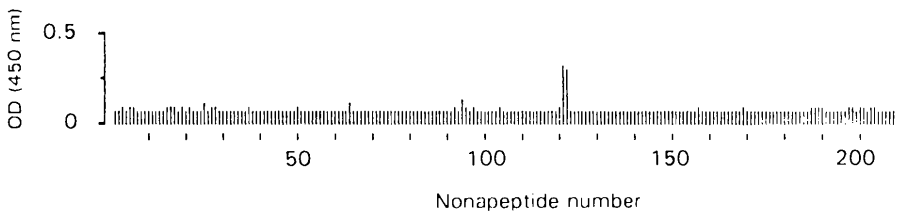

Fig 1. PEPSCAN analysis with monoclonal antibody 4B7 on a complete set of overlapping nonapeptides derived from the amino-acid sequence of the surface protein Pfs25 from *Plasmodium falciparum* sexual stages. The peptides are numbered according to their amino acid on the *x* axis; each vertical line represents the absorbance (expressed in optical density units, *y* axis) measured for a certain peptide. At 1:1000 dilution MAb 4B7 reacted specifically with two consecutive nonapeptides 121 and 122, ILDTSNPVK and LDTSNPVKT with light absorption values 0.324 and 0.312, respectively, while the other 209 peptides had an average value of 0.092.

Synthetic peptides for crystallization

Peptides and peptide analogues corresponding to Ile122 -Val132 from Pfs25 were prepared by solid-phase peptide synthesis. Each linear peptide was blocked at the N terminus by acetylation and at the C terminus by amidation upon cleaving from the resin. Linear peptides were synthesized with an Advanced ChemTech 350 multiple peptide synthesizer on Rink amide resin (NovaBiochem, 0.46 meq g^{-1}) using a standard Fmoc synthesis strategy where amino acids were coupled with diisopropylcarbodiimide and hydroxybenzotriazole. The side chains of Fmoc amino acids were protected by butyl (Asp, Thr, Ser), Boc (Lys) or trityl (His) groups. Cyclic peptides were synthesized on solid support using glycine analogues (Arrhenius & Satterthwait, 1990) to from a hydrazone link as has been described by Chiang, Cabezas, Calvo & Satterthwait (1994). Peptides were cleaved from the resin and protecting groups removed by treatment with trifluoroacetic acid:water (95:5) for 1 h and precipitated with ether. Each peptide was purified to >98% homogeneity on an RP-18 column (Vydac 201TP1022, 2.2 × 25 cm, 10 μm particles) using HPLC and a 0–100% water/acetonitrile gradient in 0.1% trifluoroacetic acid. Each peptide was identified by FAB mass spectroscopy.

Crystallization procedures

Reagents for crystallization were ammonium sulfate, sodium phosphate, potassium phosphate and sodium citrate from Fisher Scientific, polyethylene glycols (PEGs) 600, 4000 and 10 000 from Fluka Chemical Corporation and PEG 8000 from Baker Chemical Company, and imidazole and malic acid from Sigma Chemical Company. Imidazole malate stock solution was made by mixing solutions of 2 *M* imidazole and 2 *M* malic acid to achieve the desired pH. This solution was subsequently diluted tenfold to buffer the PEG solutions. Sodium citrate, NaH_2PO_4, K_2HPO_4 and sodium borate were used to buffer salt solutions depending on the required pH.

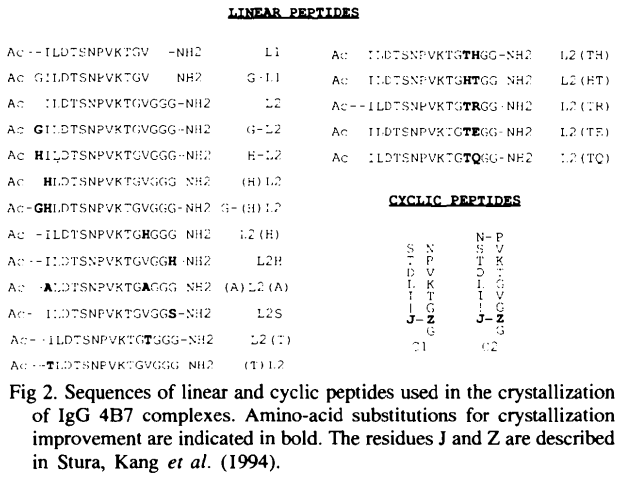

Fig 2. Sequences of linear and cyclic peptides used in the crystallization of IgG 4B7 complexes. Amino-acid substitutions for crystallization improvement are indicated in bold. The residues J and Z are described in Stura, Kang *et al.* (1994).

Crystallization conditions were screened, and crystals were grown by the vapor-diffusion method [see McPherson (1982) and Ducruix & Giegé (1992) for general crystallization methodology], using multiwell sitting-drop vapor-diffusion plates (Stura, Johnson *et al.*, 1989; Stura & Wilson, 1990, 1991). The screening consisted of a limited initial search or footprint (Stura, Nemerow & Wilson, 1992) with ammonium sulfate, mixed NaH_2PO_4 and K_2HPO_4, sodium citrate, and PEGs of molecular weights 600, 4000 and 10 000. Crystallization conditions were determined for free IgG and its complexes with various peptides (Fig. 2). In addition, samples corresponding to the individual fractions separated by ion-exchange purification underwent independent crystallization trials to assess the benefits of such purification on the ability of the IgG preparation to crystallize. Extensive use of seeding techniques was essential both to determine the optimal crystallization conditions and to produce large crystals by macroseeding (Stura & Wilson, 1990, 1991). Sitting drops consisted typically of 2.5 μl of protein from 5 to 20 mg ml^{-1} in 100 m*M* sodium acetate buffer pH 5.0 to which the same volume of reservoir solution was added. The completed trays were allowed to equilibrate overnight to up to 5 d prior to seeding in a constant temperature incubator at 295.5 K.

X-ray data collection

The crystals were mounted in thin-walled glass capillaries and data were collected using a Siemens proportional counter multiwire area detector mounted on an Elliott GX-18 rotating-anode X-ray generator operating at 40 kV and 55 mA using a 100 μm focal cup and Franks focusing mirrors (Harrison, 1968). A crystal-to-detector distance of 30 cm was needed to separate the reflections and a helium cone designed and constructed in our laboratory was used to reduce background scatter and absorption which can seriously attenuate the signal at this crystal-to-detector distance.

Results and discussion

Crystallization of IgG 4B7–peptide complexes

IgG obtained after the initial size-exclusion purification yielded polycrystalline aggregates from the footprint screening (Stura, Nemerow & Wilson, 1992). Screens, for comparison, were also set up for its complex with a linear peptide, ILDTSNPVKTGVGGG (L2) and with the corresponding closed loop (C2) (Fig. 2). The crystallinity of the round aggregates (polycrystal clumps) obtained for the uncomplexed IgG was assessed on the basis of their ability to streak seed. Initial crystallization conditions were obtained by comparing solubilities of the IgG–peptide complex and the free IgG. It has been noted in the crystallization of several Fab–peptide complexes that the solubility of the complex, relative to the uncomplexed Fab, can provide an indication of the proximity to conditions suitable for nucleation and

crystal growth (Schultze-Gahmen *et al.*, 1988; Stura, Stanfield *et al.*, 1989, 1992). Common manifestations are differences in solubility and in the morphology of the precipitate produced by each. In this case, the uncomplexed IgG differed in its ability to produce round aggregates from the peptide complex in most of the footprint conditions with polyethylene glycol as a precipitant. These conditions were expanded in a finer grid search and by testing additives. A crystalline-looking aggregate was obtained from 12% polyethylene glycol 4000, 250 m*M* NaCl, 200 m*M* imidazole malate, pH 5.5. The crystallinity was verified using the streak-seeding technique yielding plate crystals. Either streak seeding or macroseeding were used to obtain crystals of a size suitable for X-ray diffraction studies (see Fig. 3*a*).

Crystallization experiments were also set up to identify any difference that existed between the material obtained from the peak and from that of the shoulder fractions. While crystals from the peak were generally of better quality than those from the shoulder, because of the low yield of peak (25%) from the purification, crystallization trials were performed mainly with shoulder material. With this strategy it was possible to reserve the use of peak material to grow crystals for X-ray data collection, once optimal conditions were determined using less pure, but more plentiful material.

Precipitants

Both salts and polyethylene glycol (PEG) were found to be effective precipitants for both the uncomplexed and peptide-bound IgG. Polycrystals composed of extremely thin hairlike needles were obtained for the uncomplexed IgG and for many of the complexes. This dominant crystal form was unsuitable for crystallographic studies. Plate crystals were obtained with PEG of molecular weights (MWs) 3000 to 10 000 without seeding and as low as 2000 with seeding. In the lower MW PEG (1000-2000) experiments a gel phase was observed at the bottom of the sitting drop. Crystallization could be induced by seeding, and crystals grew more slowly than with the higher MW PEGs (3000-10 000). The advantage of using lower MW PEGs was that no unwanted polycrystals formed spontaneously compared with higher MW PEGs. Polycrystals were formed in PEG 4000 after 3-5 d, leaving time for introducing plate-crystal seeds. By seeding the experiments within 1-2 d the problem of polycrystal formation was minimized. With PEG 2000, no polycrystals are produced but the resulting growth rate of the plate crystals was slower by a factor of two compared with higher MW PEGs. The influence of KCl, NaBr, sodium benzoate, NaH_2PO_4 and K_2HPO_4 as alternative salts to NaCl was analyzed. The crystals obtained with KCl showed some improvement over those with NaCl as an additive. No significant effect was noted for organic additives such as methylpentane diol (MPD) and ethanol.

Cross-seeding

The relative solubility of the various IgG-peptide complexes studied was monitored by footprint experiments and by a more limited screen of conditions close to those for the plate crystals of the IgG4B7-L2 complex, specifically 8-14% PEG 4000, 200 m*M* imidazole malate, 250 m*M* NaCl, pH 5. 5. These experiments were cross-seeded by the streak-seeding technique with crystals of the IgG4B7-L2 complex after 1-2 d pre-equilibration. In many cases, cross seeding resulted in a positive response. Some generalizations can be drawn from the results: in particular, addition of glycine at the N-terminus of the peptide [G-L1, G-L2, G-(H)-L2], changed the crystallization properties of the complex; polycrystal clumps were not observed for these peptide complexes; seeding is required and crystal growth is slow; and peptides with specific changes at the C terminus grow at a faster rate. From these results it is clear that the peptide has a strong role in the IgG-peptide complex crystallizations and it appears possible that a peptide could be designed from structural information obtained from Fab-peptide complex crystals (Stura, Kang *et al.*, 1994) to give better diffracting IgG-peptide cocrystals.

Cyclic peptides

An example of a cyclic peptide which has been synthesized for binding and crystallization studies is [N=CH—$(CH_2)_3$CO]—ILDTSNPVKTG—(NCH_2CO)G (C1; Fig. 2). Binding studies with MAb 4B7 have shown a markedly higher affinity for this cyclic peptide compared with linear peptides. Currently, only round aggregates, the dominant polycrystalline form, have been obtained for cyclic peptide-IgG complexes. Seeds from these round aggregates can be used in streak-seeding experiments to demonstrate their crystallinity. Cross-seeding from IgG-linear peptide complex crystals has not produced positive results, possibly implying that the polycrystals of the constrained peptide complex form a different reticulum from that of the linear peptide, although the crystallization conditions for the linear and the cyclic peptide-IgG complexes were virtually identical as were the solubilities under these conditions (see *Reverse Screening*; Stura, Satterthwait, Calvo, Kaslow & Wilson, 1994). In addition, crystals of the linear peptide complex dissolved when placed in a solution containing IgG and cyclic peptide equilibrated at the appropriate crystallization conditions for both. Complex crystals of the Fab cleaved with elastase are obtained spontaneously with this cyclic peptide (Stura, Kang *et al.*, 1994). Further trials with cyclic peptides are underway with the aim of obtaining better crystals of these complexes. The structure determination of the Fab-cyclic peptide complexes (Stura, Kang *et al.*, 1994) may be of use for the design of cyclic peptides for the IgG-cyclic peptide cocrystallization.

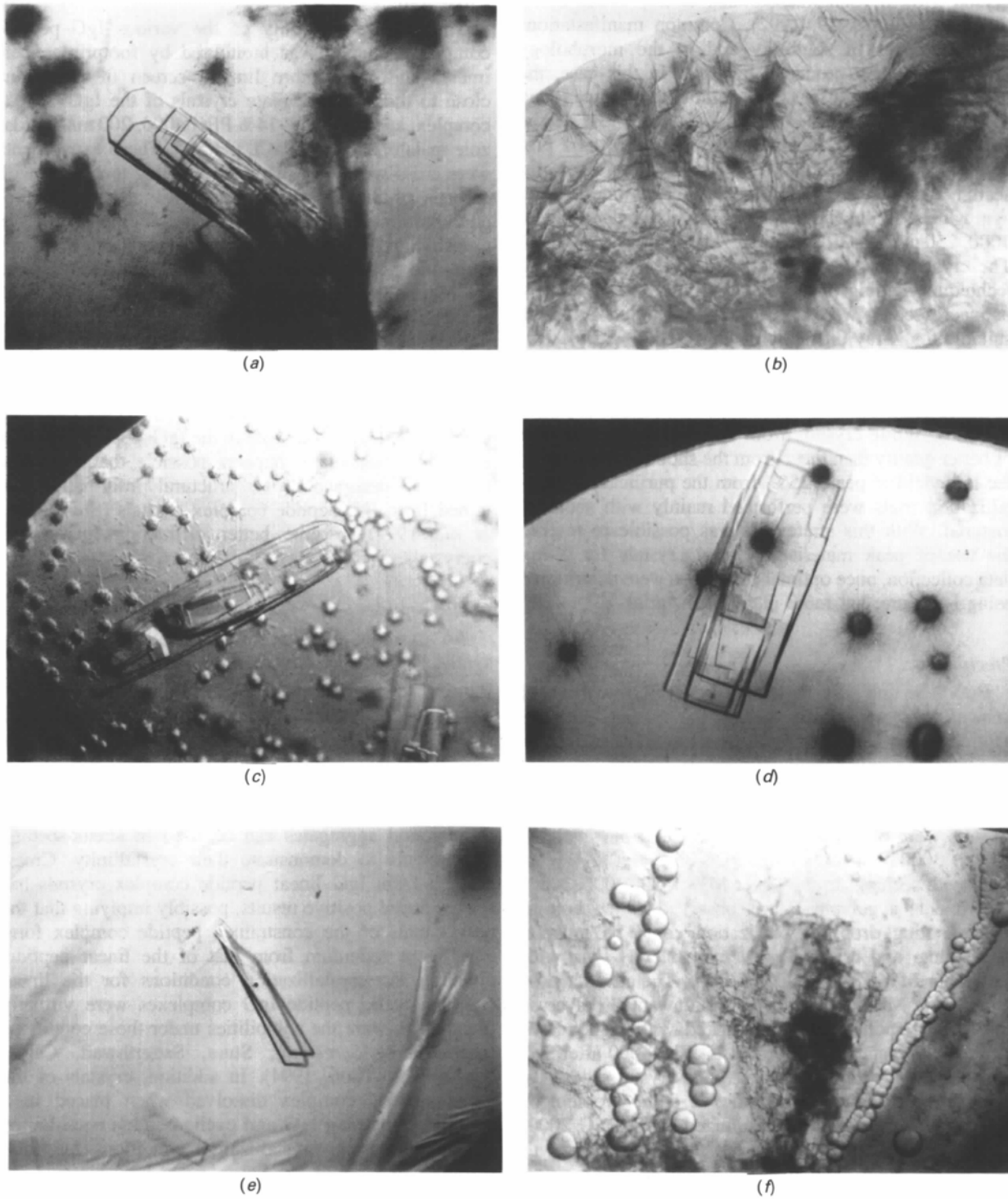

Fig 3. Photomicrographs of crystals of IgG 4B7. (*a*) Crystals of the IgG 4B7 complex with peptide L2 grown from IgG at 10 mg ml^{-1} with 12% PEG 4000, 250 m*M* NaCl, 0.2 *M* imidazole malate, pH 5.5 as a precipitant in a cooled constant temperature incubator at 295.2 K. A cluster like this can be used to demonstrate the resolution to which such crystals diffract (Fig. 3). (*b*) Microcrystals of the free IgG obtained from various salts. (*c*) Crystal plates of the complex with L2(T) have more rounded edges. Crystals with the L2(H) peptide grow more rapidly and are somewhat thicker than from other complexes. (*e*) Single crystals can be easily separated from clusters like these. (*f*) Round aggregates capable of nucleating further aggregates are shown here in a streak-seeding experiment. In general crystallization conditions are within the range 10–16% PEG 4000, 0–250 m*M* NaCl, 50–200 m*M* imidazole malate, pH 5.5–6.0. A similar range has been observed for the Fab 4B7 crystallization but with a pH range 5.5–8.5 (Stura, Kang *et al.*, 1994).

Preliminary X-ray studies

Both thin-plate single crystals (Figs. 3*a*-3*e*) and an agglomerate of many closely aligned stacked crystals of IgG 4B7-peptide complex (see Fig. 3 for individual crystallization conditions) were analyzed. The single crystals were found to be too thin to produce a signal that could be measured above the background. Crystal clusters in which the individual plates were aligned closely enough that reflections from the individual crystal plates layers become superimposed were used to demonstrate that the crystals are ordered to at least 5 Å (see Fig. 4). The pattern produced by the cluster resembles the pattern that could be obtained in a screenless precession photograph. This approach was useful to screen crystals for diffraction to determine the resolution limit without committing large amounts of protein and effort to growing single crystals. While the approach is likely to underestimate the extent to which single crystals of equivalent size to the cluster would diffract, it nonetheless provided a valuable tool for the early analysis of crystals during crystallization improvement studies. The current crystals are only suitable for determining the overall arrangement of the Fab and Fc domains, although they will be able to give a full description of the IgG structure when combined with the Fab complex structures.

The sequences for the variable domains have now been determined. A molecular-replacement solution has been obtained for the elastase-cleaved Fab 4B7 free and complexed with cyclic peptides (Stura, Kang *et al.*, 1994). The structure of the Fab-peptide complex may provide indications for the crystallization of the IgG and its complexes.

The problem with this IgG crystallization lies in the competition between many crystal forms which can grow from closely related crystallization conditions. This is the case also for the Fab-peptide complexes, but since spontaneous nucleation is rare, seeding can be used to make the crystallization easier to manage. Because the IgG-peptide complex crystals nucleate spontaneously in an undesirable form, seeding with the plate form must be performed within 2 d. However, the growth of the crystals within the seeded drop does not reduce the degree of supersaturation sufficiently to prevent the eventual nucleation of the polycrystals. Although this can be considered only a partial success, what has been learned from this work will contribute to the development of new ideas and procedures for improving the current crystallization methodology for IgGs.

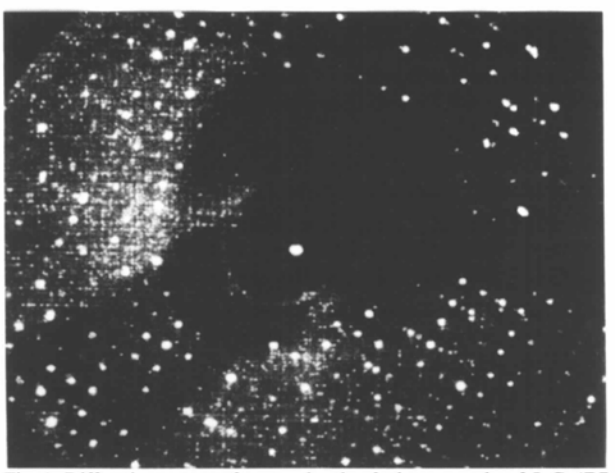

Fig 4. Diffraction pattern from a sheath of plate crystals of IgG 4B7 complexed with peptide L2.

Concluding remarks

Crystallization of whole immunoglobulins or of $F(ab')_2$ may present new possibilities in studies of antibodies and their complexes which have concentrated mainly on the study of Fab or Fv. Progress has been slow and difficult both with IgG and $F(ab)_2$, and while crystals for this IgG and of an $F(ab')_2$ have been obtained in our laboratory neither diffract significantly beyond 4 Å, which makes them of limited use for crystallographic studies. Similarly, slow progress has been made with IgM to yield crystallizable Fab fragments (Newkirk, Edmunson, Wistar, Klapper & Capra, 1987), although to date no three-dimensional structure of such fragments have been reported. Crystallization of whole immunoglobulins and their antigen and receptor complexes are likely to remain somewhat troublesome, nevertheless efforts should continue to be made to obtain structural information on antigen-whole antibody interactions and on how antibodies may interact with C1q or the Fc receptor.

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