The action of pepsin on porcine immunoglobulin M and its effect on biological activity

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Treatment of porcine immunoglobulin M (IgM) with pepsin at pH 4.6 and 37°C was found to gradually remove Fab arms and C μ 2 domains over a period of 18 h. Structural studies failed to find any other change. The main products can therefore be regarded as IgM-like molecules with limited numbers of Fab arms and C μ 2 domains. Results indicated that this removal of Fab arms is probably a random process. As the average number of Fab arms per molecule was decreased the ability to agglutinate *Salmonella oranienburg* (mt-H) gradually diminished. Complement fixation by the complexes however, decreased rapidly, and became negligible when the average number of Fab arms was four. This was confirmed by using a preparation containing mainly molecules with three or four Fab arms. The overall results showed that molecules with three or four Fab arms can agglutinate *Salmonella* but that these complexes do not fix complement. Molecules with five arms probably behave like those with four. Complexes formed by molecules with six arms fix complement quite efficiently. Possible explanations for these results are discussed.

Various aspects of the structure and function of IgM have been dealt with in numerous reviews (Metzger, 1970, 1974, 1978; Porter, 1973; Nisonoff *et al.*, 1975; Beale & Feinstein, 1976; Feinstein & Beale, 1977). In brief, the mammalian IgM molecule is a cyclic, disulphide-linked pentamer in which each monomer consists of two light (L) and two heavy (μ) polypeptide chains. Each molecule also usually contains a J-chain (for a review see Koshland, 1975).

The L-chain is folded into two conformationally related globular domains V_L and C_L (Schiffer *et al.*, 1973; Poljak *et al.*, 1973) and the μ -chain has five homology regions (Putnam *et al.*, 1973; Watanabe *et al.*, 1973) which are believed to fold into five conformationally related domains, V_H, C μ 1, C μ 2, C μ 3 and C μ 4 (see Beale & Feinstein, 1976). In gross shape the IgM molecule can be regarded as having a central, disc-like, (Fc)₅ portion (containing the C μ 3 and C μ 4 domains) from which protrude five pairs of arm-like Fab portions (containing V_L, C_L, V_H and C μ 1 domains). These are linked to the (Fc)₅ portion by globular C μ 2 domains (see Fig. 1).

Each Fab portion contains an antigen binding site whereas the (Fc)₅ portion contains a complement

fixation site. The antigen binding sites are thus remote from the complement fixing site and the mechanism whereby antigen binding activates the complement site has been the subject of much investigation, discussion and speculation (see Metzger, 1974, 1978; Feinstein & Beale, 1977).

The method of enzymic fragmentation of immunoglobulin (Porter, 1959) has been extensively applied to the study of IgM (see Metzger, 1970, 1974). It has been reported (Beale, 1974*a,b*) that pig IgM is more resistant to proteolysis than is the more widely used human IgM. This allows the gradual removal of Fab arms and C μ 2 domains over a period of some 18 h by means of pepsin (Beale & Kent, 1978).

When purified material from a 4 h fragmentation was examined it appeared to consist of IgM-like molecules having an average of only four Fab arms per molecule instead of ten (Beale & Kent, 1978). This material agglutinated particulate antigen almost as efficiently as the original IgM antibody, but the complexes no longer fixed whole complement (Beale & Kent, 1978).

The production of such molecules is of considerable interest since their study could provide important information about the structural criteria required for antigen binding to initiate complement fixation.

Abbreviations used: IgM (etc.), immunoglobulin M (etc.); SDS, sodium dodecyl sulphate.

In the present paper we have studied the action of pepsin in some detail and present results that strongly suggest that removal of Fab arms from IgM is largely a random process. The structures of the molecular products have been extensively examined and they prove to be IgM-like molecules that have been modified only by the removal of specific numbers of Fab arms and C μ 2 domains. There appears to be a definite relationship between the number of arms on a molecule and its ability to fix complement when complexed with particulate antigen.

Experimental

Materials

Pepsin and carboxypeptidase-A were obtained from Worthington (Freehold, NJ, U.S.A.); dithiothreitol and iodoacetamide from Sigma Chemical Co. (Poole, Dorset, U.K.); guanidine carbonate, AnalaR grade, from Hopkin & Williams (Chadwell Heath, Essex, U.K.); Bio-Gel A-5m and P-300, acrylamide and bisacrylamide (electrophoresis grade) from Bio-Rad Laboratories (Richmond, CA, U.S.A.); Sephadex G-200 and polyacrylamide gradient gels from Pharmacia (London W5, U.K.); DEAE-cellulose (DE-23) from Whatman (Springfield Mill, Maidstone, Kent, U.K.). Complement fixation test diluent tablets were from Oxoid, and rabbit haemolytic serum and *Salmonella* mt-H suspension was from Wellcome Reagents, (Beckenham, Kent, U.K.).

Methods

Preparation of IgM. The method of Chaplin *et al.* (1965) was used. A euglobulin ppt. was prepared from serum and lipoprotein was removed by preparative ultracentrifugation at 106 000 *g* for 22 h. The crude IgM was purified by chromatography on DE-23 and gel filtration through Sephadex G-200 or Bio-Gel A-5m. The purity was examined by immunoelectrophoresis, SDS/polyacrylamide-gel electrophoresis, gradient-gel electrophoresis and analytical ultracentrifugation. Sera from normal pigs and pigs injected with formalized *Salmonella* mt-H were used.

Fragmentation with pepsin. The method has already been fully described (Beale, 1974*a,b*; Beale & Kent, 1978). Solutions of IgM (0.5–1.0%, w/v, in 0.2 M-NaCl/0.1 M-sodium acetate, pH 4.6) were digested with pepsin (protein:enzyme, 100:1, w/w) at 37°C for various times between 0.5 and 24 h. After adjusting them to pH 6.0, the samples were examined by analytical ultracentrifugation, SDS/polyacrylamide-gel electrophoresis and gradient-gel electrophoresis. They were then gel filtered through Sephadex G-200 or Bio-Gel A-5m

and the purified products were examined by the above methods.

Analytical ultracentrifugation. This was carried out as previously described (Beale & Feinstein, 1969) in standard buffers with a Beckman model E analytical centrifuge fitted with Schlieren optics. The relative amounts of sample components were determined by area integration of the Schlieren patterns with correction for radial dilution. Measurement of duplicate samples and of known mixtures of Fab and (Fc)₂ fragments showed that the error of this method lay within $\pm 5\%$. Values obtained for sedimentation coefficients were reproducible to $\pm 2\%$.

SDS/polyacrylamide-gel electrophoresis. The technique of Summers *et al.* (1965) was used. Samples, in 0.1 M-sodium phosphate buffer, pH 7.2, containing 0.5 M-urea and 0.1% (w/v) SDS, were run in 4.5% (w/v) gels (8 cm $\times$ 0.5 cm) at a constant current of 10 mA/gel for 3–4 h at 20°C. The gels were then stained for 1 h at 20°C in 0.1% (w/v) Amido Black in fixer (ethanol/acetic acid/water, 25:8:67, by vol.), and then destained in fixer at 20°C. A Quick-Scan Junior (Helena Laboratories, Beaumont, TX, U.S.A.) was used for densitometric scanning of gels. Measurements on duplicate samples of known mixtures of μ -chain, L-chain and Fc showed that the error of this method lay within $\pm 10\%$.

Gradient polyacrylamide-gel electrophoresis. The method is similar to that described by Margolis & Kenrick (1967), except that ready-made Pharmacia 2–16% (w/v) polyacrylamide gels were used. The gels were pre-run for 15 min at 120 V before sample loading. After sample loading, 50 V was applied for 30 min and then electrophoresis was carried out at 120 V for 18–23 h at 15–16°C. The running buffer was 0.17 M-Tris/0.024 M-boric acid/0.007 M-EDTA, pH 8.8. Gels were stained at 37°C for 1 h with 0.2% (w/v) Coomassie Blue in fixer (methanol/acetic acid/water, 3:2:11, by vol.) and destained in fixer at room temperature. Densitometric scanning was then carried out.

Reduction and alkylation. This was carried out as previously described (Beale & Feinstein, 1969). Samples (0.5–1.0%, w/v) in 0.2 M-NaCl/0.1 M-Tris/HCl, pH 8.0, were made 10 mM with respect to dithiothreitol and allowed to incubate at room temperature for 1 h. Sufficient iodoacetamide or iodoacetic acid was then added to give 25 mM in the reaction mixture. Some samples in 6 M-guanidinium chloride were reduced with 20 mM-dithiothreitol and alkylated with 60 mM-iodoacetamide.

Separation of peptide chains. Samples that had been reduced and alkylated in 6 M-guanidinium chloride were applied to a column of Bio-Gel P-300 (1.6 cm $\times$ 70 cm) and eluted with 6 M-guanidinium

chloride/acetic acid (pH4.0). Eluted protein was detected by absorption at 280nm. The purity of the separated components was checked by SDS/polyacrylamide-gel electrophoresis.

Amino acid analysis. Samples (0.1 mg) of purified μ -chain were hydrolysed with constant-boiling glass-distilled HCl *in vacuo* at 108°C for 24h. Amino acid analysis was then carried out on a modified Technicon Microanalyser (Beale & Kent, 1968).

Peptide mapping. Samples (2–5 mg) of purified μ -chain were digested with trypsin (protein:enzyme, 50:1, w/w) in 0.1M-NH₄HCO₃, pH8.5, for 16h at 37°C. Peptides were separated by high-voltage paper electrophoresis in pyridine/acetic acid/water (1:10:89, by vol.), pH3.5, followed in the second dimension by chromatography in butan-1-ol/acetic acid/pyridine/water (15:3:10:12, by vol.). Peptides were revealed with 0.2% (w/v) ninhydrin in acetone.

Agglutination of *Salmonella* and complement fixation by complexes. Aliquots (0.1 ml) of a 2-fold serial dilution of the purified sample having anti-*Salmonella* activity were mixed with aliquots (0.1ml) of a 2-fold serially diluted *Salmonella* suspension. After incubation at 37°C for 4h the samples were examined visually for agglutination. Complement fixation studies were then carried out by the micro-method of Wasserman & Levine (1960) using pig serum, absorbed at 0°C on sheep erythrocytes, as the source of complement. To each 0.2ml of complexed sample was added 0.8ml of complement-fixation-test diluent and 0.2ml of a pre-determined dilution of complement sufficient to give complete lysis of a standard number of optimally sensitized sheep erythrocytes. After incubation for 1h at 37°C 0.2ml of a 0.25% suspension of sensitized sheep erythrocytes was added. After a further incubation at 37°C for 1h the lysis in each sample was measured by determining its absorbance at 413nm. The usual controls, as detailed by Wasserman & Levine (1960), were included in each assay. Assays were generally done on duplicate samples and repeated two or three times. End points were reproducible to within a single tube of a 2-fold serial dilution.

C-terminal analysis. This was carried out on purified μ -chains by the method of Ambler (1967) by using carboxypeptidase A.

Results

Examination of digestion products by analytical ultracentrifugation and SDS/polyacrylamide-gel electrophoresis

Several series have been examined and Table 1 shows a typical set of results. Throughout a digestion series only two components were revealed by analytical ultracentrifugation or gel filtration. One of these sedimented at 3.5S and had immuno-

Table 1. Examination of digestion products by analytical ultracentrifugation and SDS/polyacrylamide-gel electrophoresis and measurement of biological activity
To measure biological activity, samples having the same absorbance at 280 nm were 2-fold serially diluted.

Sample	Fab in digest (%)	S _{20,w} at 0.5% concn. of high-mol.-wt. product (S)	Composition (%) by SDS/polyacrylamide-gel electrophoresis of reduced and alkylated high mol. wt. product*			Average no. of Fab arms on high-mol.-wt. product	Dilution of product to agglutinate 10 ⁸ <i>Salmonella</i> at equivalence	Dilution of product (complexed with 10 ⁸ <i>Salmonella</i>) to fix at least 70% complement
			μ	Fc	L + J			
IgM incubated at 37°C, pH4.6, 6 h								
IgM, untreated	0	17.3	77	0	23	10	1:512	1:128
Peptic digest (1 h)	0	17.5	75	0	25	10	1:512	1:128
Peptic digest (2 h)	9	16.0	71	7	22	8	1:512	1:128
Peptic digest (3 h)	19	14.8	62	17	21	6	1:256	1:64
Peptic digest (4 h)	24	13.4	54	26	20	5	1:256	1:8
Peptic digest (6 h)	33	11.9	44	40	16	3-4	1:128	>1:2
Peptic digest (12 h)	40	11.5	31	59	10	2	1:32	>1:2
Peptic digest (18 h)	51	11.2	12	82	6	0-1	1:8	>1:2
Peptic digest (24 h)	55	10.9	0	100	0	0	>1:2	>1:2
	56	11.0	0	100	0	0	>1:2	>1:2

* Averaged values from the densitometric scanning of three gels that did not differ by more than 6%.

chemical properties characteristic of pig Fab fragment (Beale, 1974*a,b*) of mol.wt. 45000. As can be seen from Table 1 (column 2) the proportion of this product increased with digestion time up to 18h, after which there was little change. The other product (termed the high-molecular-weight product) displayed a decrease in sedimentation coefficient with increasing time of digestion up to 18h, after which there was very little, if any, significant change (Table 1, column 3).

SDS/polyacrylamide-gel electrophoresis of the reduced and alkylated product revealed a decreasing proportion of μ -chain and an increasing proportion of Fc with increasing digestion time up to 18h, when no μ -chain could be detected (Table 1, columns 4 and 5).

These results are compatible with a process whereby Fab arms are gradually removed from the IgM molecule to leave an (Fc)₅ fragment after 18h. This fragment has already been characterized (Zikan & Miler, 1974; Beale, 1974*a,b*). At intermediate stages of digestion the high-molecular-weight product would consist of IgM-like molecules containing limited numbers of L-chains and μ -chains which contribute towards the remaining Fab arms (see Fig. 1).

Fig. 2 shows that the molecular weights of L-chain and μ -chain in the high-molecular-weight product remain the same within the limits of the technique of SDS/polyacrylamide-gel electrophoresis. The L-chain appears as two bands due to

the presence of κ and λ types, of which the latter behave abnormally (Virella & Coelho, 1974).

The Fc component from the high molecular weight product is represented by three bands in Fig. 2. During the early stages of digestion there is very little or no change in these bands, but a pronounced alteration in relative intensity occurs between 6 and 12h. The exact relationship between these three bands has yet to be fully established but they are most probably due to peptic cleavage at different, but nearby, sites in the μ -chain. These sites appear to be separated by only a few amino acid residues and one is probably more readily cleaved than the other. The three bands were integrated together when measuring the proportion of Fc and the change in relative intensities in later stages of digestion was regarded as insufficient to seriously influence the measurement of the composition of the high-molecular-weight product.

The central Fc band corresponds to the position of a reduced and alkylated human tryptic (Fc)₅ marker prepared by the method of Plaut & Tomasi (1970). The structure of this fragment is known in considerable detail (Putnam *et al.*, 1973) and does not contain the C μ 2 domain. The results from SDS/polyacrylamide-gel electrophoresis, gradient polyacrylamide-gel electrophoresis (described below) and electron microscopy (Feinstein *et al.*, 1976), indicate that pig (Fc)₅ has a similar size to that of human (Fc)₅ and hence does not contain the C μ 2 domain. It therefore seems that the removal of an

Fig. 1. Action of pepsin on pig IgM

Pig IgM is a cyclic pentamer, but only one monomer is shown. The structure is based on the one already established for human IgM. Domains are represented by rectangles and disulphide bridges by dashed lines. AB, antigen binding site, location well established. CF, complement-fixing site, lies in the Fc fragment but the exact location is uncertain.

Fab arm from pig IgM is accompanied, or quickly followed, by loss of the C μ 2 domain (Fig. 1).

Calculation of the average number of Fab arms per molecule of high-molecular-weight product

Table 2 gives theoretical values for the amount of Fab released and for the μ -chain, L-chain and Fc

composition of the high-molecular-weight products after the hypothetical removal of specific numbers of Fab arms and C μ 2 domains. In the calculation of these values the molecular weights of pig μ -chain and pig Fc were assumed to be 73 500 and 35 600 respectively, the same as values derived from the amino acid sequence (Putnam *et al.*, 1973; Watanabe *et al.*, 1973) and glycopeptide compositions (Shimizu *et al.*, 1971) of human μ -chain and Fc. It is already known that the primary structure of canine μ -chain (McCumber & Capra, 1979) and murine μ -chain (Kehry *et al.*, 1979) are closely related to that of human μ -chain. Hence it can be assumed that porcine μ -chain will also be closely related. Molecular weights of 22 500 and 45 000 have been used for L-chain and Fab fragment respectively, which are average literature values.

When the experimental values shown in Table 1 (columns 2, 4, 5 and 6) are compared with the theoretical values given in Table 2 it is possible to assess the average number of Fab arms per molecule of high-molecular-weight product at various stages of the digestion series. These are shown in column 7 of Table 1. As might have been expected, the average number of Fab arms clearly decreases with increasing digestion time. Incidentally, theoretical values based on the removal of Fab arms only (results not shown) have a much greater divergence from the experimental values given in Table 1 than when theoretical values are used based on the removal of Fab arms and C μ 2 domains (Table 2). This in itself indicates that the latter process is the most probable.

Examination of high-molecular-weight products by gradient polyacrylamide-gel electrophoresis

A typical set of results is shown in Fig. 3. In this particular experiment samples were taken at 0.5, 1, 1.5 and 2 h from a 37°C digestion. The digest tem-

Fig. 2. SDS/polyacrylamide-gel electrophoresis of reduced and alkylated samples (a) IgM; (b) 3 h digestion product; (c) 6 h digestion product; (d) 12 h digestion product. ➡, Position of human tryptic Fc marker.

Table 2. Theoretical values for products formed by the removal of specific numbers of Fab arms and C μ 2 domains from pig IgM

The following molecular weights were used in the calculations: IgM, 975 000; (Fc)₂, 356 000; Fab, 45 000; μ -chain, 73 500.

Fab arms and C μ 2 domains removed	Fab in digest (%)	Number of Fab arms remaining on high-mol.-wt. product	Composition of high-mol.-wt. product (%)		
			μ	Fc	L + J
0	0	10	75.4	0	24.6
1	4.7	9	72.4	3.9	23.7
2	9.5	8	68.8	8.4	22.8
3	14.5	7	64.8	13.4	21.8
4	19.7	6	60.2	19.4	20.4
5	25.0	5	54.5	26.5	19.0
6	30.6	4	48.0	34.9	17.1
7	36.3	3	40.0	44.9	15.1
8	42.3	2	29.2	57.9	12.2
9	48.4	1	17.1	74.2	8.7
10	54.8	0	0	100	0

Fig. 3. *Gradient polyacrylamide-gel electrophoresis*

(1) IgM; (2–12) high-molecular weight products from peptic digestion of IgM at the following temperatures and times: (2) 30 min, 37°C; (3) 1 h, 37°C; (4) 1 h 30 min, 37°C; (5) 2 h, 37°C; (6) 2 h 20 min, 37°C; 1 h 10 min, 20°C; (7) 2 h 50 min, 37°C; 1 h 10 min, 20°C; (8) 3 h 20 min, 37°C; 1 h 10 min, 20°C; (9) 4 h, 37°C; 2 h 30 min, 20°C; (10) 5 h, 37°C; 2 h 30 min, 20°C; (11) 5 h 55 min, 37°C; 2 h 30 min, 20°C; (12) 24 h, 37°C. (13–15) Bio-Gel A-5m fractionation of sample 9: (13) pool 1; (14) pool 2; (15) pool 3.

perature was then lowered to 20°C for 70 min while it was examined on the analytical ultracentrifuge. Digestion was then continued at 37°C and further samples were taken. The temperature was again lowered to 20°C for a second examination on the analytical ultracentrifuge and then continued at 37°C once more.

The most striking feature of Fig. 3 is the system of bands, most of which are clearly resolved. By careful examination and measurement of several gels we have been able to account for 11 bands (marked at edge of gel) although bands 8, 9 and 10 are badly resolved. The band system starts in coincidence with an IgM marker and finishes slightly short of an (Fc)₃ marker obtained from a 24 h digest of pig IgM. The change of spacing between the bands is due to the curved gradient of the gel.

This system of bands is best explained by a series of molecules that differ from each other by one Fab arm and one Cμ2 domain to give an overall total of eleven species having from zero to ten Fab arms. The bottom band of the system represents an (Fc)₃ fragment which has a slightly higher molecular weight than (Fc)₃ fragment obtained from a 24 h digest. Evidence for such a difference has been presented above and is shown in Fig. 2.

A further point of interest seen in Fig. 3 is that the band patterns of the samples gradually shift from higher to lower molecular weight regions of the gel (from top to bottom) with increasing length of digestion and there are accompanying changes in band intensities. These results are compatible with a process of random removal of Fab arms and

Cμ2 domains. Indeed, densitometric scanning of the gels showed that the band intensities agreed quite well with theoretical frequencies calculated from a mathematical model for the random removal of Fab arms (Beale & Kent, 1978).

Preparation of a high-molecular-weight product with a restricted number of molecular species

It was arranged that sample 9 of Fig. 3 should be large enough for gel filtration on a column (Pharmacia K26/100) of Bio-Gel A-5m. When this was done a broad peak was obtained. The leading, central and trailing sections of this peak were made into pools 1–3 respectively and were concentrated by pressure dialysis. The pools were examined by gradient polyacrylamide-gel electrophoresis to give the results shown in Fig. 3 (samples 13–15). Pool 1 from the column (sample 13 in Fig. 3) clearly consisted of a population of restricted molecular species containing almost entirely three, four and five-arm molecules with traces of molecules with two and six Fab arms. Pools 2 and 3 (samples 14 and 15 in Fig. 3) were more heterogeneous.

Effect on biological activity and its relationship to the average number of Fab arms per molecule

Several digestion series have been examined and a typical set of results is shown in Table 1 (columns 8 and 9). In this particular experiment samples having the same absorbance at 280 nm were serially diluted and mixed with serial dilutions of *Salmonella*, but only the values for one dilution of *Salmonella*

are given in Table 1. All values were reproducible to within a single tube of a 2-fold serial dilution.

It will be seen from Table 1 that an IgM control (Table 1, row 1) heated at 37°C (pH 4.6) for 6 h has the same ability to agglutinate *Salmonella* as untreated IgM (Table 1, row 2) and complement fixation by the complexes is the same. As the digestion time increases, the ability of the high-molecular-weight product to agglutinate *Salmonella* very gradually falls, but complement fixation by the complexes diminishes at a much greater rate. These changes are clearly related to the average number of Fab arms per molecule (Table 1, column 7). Thus the 1 h product, having an average of eight Fab arms, shows no difference in activity relative to the untreated IgM. The 2 h product (Table 1, row 4) shows only a marginal difference; 2-fold for agglutination and 2-fold for complement fixation. The 3 h product, having an average of five Fab arms shows only a 2-fold difference in agglutination relative to untreated IgM but there is now a very significant (16-fold) difference in complement fixation by the complexes. For the 4 h product, having an average of three to four Fab arms, there is a 4-fold difference in agglutination but no complement fixation by the complexes can be detected. At longer digestion times the ability of the high-molecular-weight product to agglutinate *Salmonella* decreases rapidly and is completely lost at 18 h, when the product is an (Fc)₃ fragment.

Relationship between gradient polyacrylamide-gel bands and biological activity

Samples shown in Fig. 3 having the same absorbance at 280 nm were serially diluted 2-fold and complexed with *Salmonella*. For sample 7, in which bands 3, 4 and 5 are the most intense, there was a 2-fold difference in agglutination relative to sample 2 (bands 8–10 most intense) but a 32-fold difference in complement fixation. Sample 8, which has bands 3 and 4 as the most intense, showed a 4-fold difference in agglutination but no complement fixation could be detected. This implies that molecules with three and four Fab arms can agglutinate *Salmonella* quite effectively but the complexes are very inefficient at fixing complement. This point is emphasized by the behaviour of sample 13, which has a restricted number of bands. The great majority of molecules in this sample have three or four Fab arms. Agglutination differed only 2-fold from sample 2, but there was a 16-fold difference in complement fixation. This low level of fixing could be entirely due to the presence of some molecules with six Fab arms. Indeed, in sample 6, molecules having six arms form one of the most intense bands and complexes of this sample fixed complement fairly efficiently. These results correlate well with those described above, which show that complement

fixation becomes very inefficient as the average number of Fab arms per molecule reaches about four.

Attempts to find structural differences between the μ -chain from a high molecular product and from IgM

μ -Chains were separated from IgM and from a high-molecular-weight product having an average of four Fab arms. No significant difference was found by amino acid analysis or was revealed by two-dimensional mapping of the tryptic peptides. The C-terminal amino acid was found to be a tyrosine residue in both cases. We have already pointed out above that the molecular-weight of the μ -chain does not change during a digestion series within the limits of the technique of SDS/polyacrylamide-gel electrophoresis.

Discussion

Structure of the high-molecular-weight products

Electron microscope studies (Feinstein *et al.*, 1976) have revealed that pig (Fc)₃ from an 18 h peptic digest of IgM has a cyclic structure. This (Fc)₃ fragment has dimensions similar to the central disc-like part of the IgM molecule. Hence pepsin digestion does not disrupt the cyclic nature of these molecules.

Under the electron microscope pig peptic (Fc)₃ fragment is seen to have a similar structure to human tryptic (Fc)₃ and therefore supposedly contains C μ 3 and C μ 4 domains but not C μ 2 domains. This is supported by results given in the present paper, which show that the pig and human (Fc)₃ fragments have similar molecular weights.

The present paper also shows that, at intermediate stages of digestion, molecules contain limited numbers of L-chains and μ -chains. Amino acid analysis, C-terminal analysis and peptide mapping failed to reveal any difference between μ -chain from a 4 h product and from untreated IgM.

The high-molecular-weight products from the limited pepsin digestion of pig IgM can therefore be regarded as IgM-like molecules that have been modified only by the removal of limited numbers of Fab arms and C μ 2 domains.

Process of Fab arm removal

There are of course several possible processes. Perhaps the most likely ones are a random process in which any arm can be removed at any time, and a paired process in which two arms are removed from the same subunit in preference to removing arms from different subunits. In a previous paper (Beale & Kent, 1978) a mathematical model for the random removal of arms was presented. In this model the rate of reaction of a particular species

was assumed to be proportional to the number of arms on that species.

In the present paper the examination of products by analytical ultracentrifugation and SDS/polyacrylamide-gel electrophoresis (Table 1) shows that the average number of Fab arms per molecule of high-molecular-weight product decreases with increasing length of digestion. The time taken to reach an average of three to four Fab arms is four times that taken to reach an average of eight arms, and the time taken to reach an average of two arms is three times that taken to reach an average of six arms. These time ratios would fit the previously proposed model (Beale & Kent, 1978) for a random process and also a model based on the removal of pairs of Fab arms.

However, in the present paper, gradient polyacrylamide-gel electrophoresis has revealed a system of 11 bands (Fig. 3) representative of a population of molecules with zero to ten Fab arms. The intensities of the bands in the samples pass through a maximum, which gradually shifts to those bands with lower numbers of Fab arms as digestion time increases (Fig. 3). These results are compatible with the model for random removal of Fab arms, but not with a model for the removal of pairs of arms. The latter process would be expected to produce a system of only six gradient gel bands. Even if there was a partial preference for the removal of pairs of arms the bands representing molecules with even numbers of arms should be more intense than those with odd numbers of arms, but this was never observed.

Although a purely random process cannot be unambiguously proved by the results given in the present paper they strongly indicate that the process is at least largely random.

Effect on biological activity

The most striking effect observed when pig IgM is treated with pepsin is that a stage is reached where agglutination is still quite effective but the resulting complexes are very inefficient at fixing complement. This happens when the average number of Fab arms per molecule has fallen to about four (Table 1).

Gradient gel electrophoresis (Fig. 3) shows that at this stage the majority of the product consists of molecules with three, four or five Fab arms. The mathematical model (Beale & Kent, 1978) gives their respective frequencies as 22, 25 and 20%. This point is emphasized by the behaviour of the sample in which the population has been restricted almost entirely to molecules with three, four and five Fab arms (Fig. 3). This sample agglutinates *Salmonella* quite well, but the complexes showed very little ability to fix complement. Indeed the very small amount of fixing that was observed could be due

entirely to the presence of some molecules with six Fab arms.

The inference from all the results given in this paper is that molecules with three or four Fab arms can agglutinate *Salmonella* but the resulting complexes are very inefficient at fixing complement. Complexes formed by molecules with six Fab arms are quite good at fixing complement. The role of molecules with five Fab arms is less clear but their complexes would seem to be poor at fixing complement.

Possible explanations for the effects on biological activity

All our structural studies indicate that molecules with limited numbers of Fab arms will have the same (Fc)₃ portion as does untreated IgM and therefore the same complement-fixing site. Why is this site not activated when suitably modified molecules, such as those with four Fab arms, are attached to *Salmonella*?

Electron-microscope studies (Feinstein & Munn, 1969) have shown that under suitable conditions IgM molecules can adopt a 'staple-like' structure when attached to *Salmonella* and that this structure might be critical for complement fixation. Such a structure requires most of the ten Fab arms to bend sharply away from the plane of the central (Fc)₃ disc. This could result directly in the exposure of the complement site or cause a conformational change in the (Fc)₃ portion which leads to activation of the site.

It is therefore possible that this 'staple-like' structure cannot be formed by molecules with only four Fab arms. Alternatively such a structure could be formed, but a four-armed 'staple' might not sufficiently expose the complement site or not produce sufficient conformational change in the (Fc)₃ portion to activate the complement site.

In molecules with limited numbers of Fab arms the arrangement of these arms may be critical for complement fixation. This might be particularly so if complement fixation by IgM is similar to that by IgG. At least two adjacent molecules of monomeric IgG are required to activate complement (see reviews by Metzger, 1974, 1978) and IgM-like molecules may therefore require at least two adjacent μ_2L_2 monomers. In a previous paper (Beale & Kent, 1978) we emphasized the possible importance of this. In the case of a molecule with four Fab arms, only one of the 26 possible isomers will have two adjacent μ_2L_2 monomers if the process of arm removal is a random one. In the case of molecules with six Fab arms there are again 26 possible isomers, of which nine will have two adjacent μ_2L_2 monomers. There may thus be a 9-fold increase in the number of active complement-fixing molecules between these two stages.

Studies using the electron microscope, C1q binding, and samples restricted almost entirely to one molecular species might be able to distinguish between some of these possibilities.

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