Immunology 1981 **43** 171

Classes and subclasses of rat antibodies: reaction with the antigen and interaction of the complex with the complement system

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Accepted for publication 20 November 1980

Summary. Antibodies against the dinitroaminophenyl (DNAP) haptenic group were raised in outbred CFY rats using HSA or LPS as carrier. Antibodies isolated by immunoadsorbent techniques were resolved into fractions representing distinct isotypes, and the resulting fractions were tested for avidity. Subclass IgG2a was found to contain antibodies of an avidity index lower than those of other IgG subclasses or IgM. IgG2a was the only isotype detected when DNAP-LPS was used for immunization. Complexes containing defined isotypes were compared for their capacity to activate homologous complement. IgGl type antibody-containing complexes displayed a low complement activating capacity compared with those containing IgG2b, IgG2c or IgG2a. The latter subclass when complexed with antigen can thus induce complement-dependent processes in spite of a low avidity. Insoluble complexes of IgG antibodies were rapidly solubilized in rat serum (CRA phenomenon), except those containing predominantly IgG2c.

INTRODUCTION

Numerous studies have been devoted to the two-directional interaction between antigen–antibody complexes and the complement system. Under appropriate conditions the complement cascade is triggered by immune complexes, and the efficiency of triggering depends on complex size, composition and on the isotype of the antibody involved (Hyslop, Dourmashkin, Green & Porter, 1970, Rajnavölgyi, Füst, Kulics, Ember, Medgyesi & Gergely, 1978, Medgyesi, Füst, Gergely & Jaton, 1979, Klaus, 1979). During the last few years convincing results have been presented revealing the capacity of complement components to modify substantially the properties of immune complexes (Takahashi, Czop, Ferreira & Mussenweig, 1976, Kijlstra, van Es & Daha 1979, Capel, Groeneboer, Grosveld & Pondman 1977).

In this paper we report studies on the biological properties of antibodies induced in rats. The isolated specific antibodies were resolved into fractions containing a certain isotype, and thus their characteristics could be compared in the form of antigen–antibody complexes. The data presented are relevant for the understanding of the role of isotype diversity in immune response.

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0019-2805/81/0500-0171$02.00

MATERIALS AND METHODS

Animals

Outbred, 150–200 g CFY rats of either sex were used.

Antigens

Purified human serum albumin (HSA), prepared in this institute by Dr S. Zgyerka or purified human IgG was reacted with 2,4 dinitro-5-fluoro aniline (DNFA), in 0·2 M Na_2CO_3-$NaHCO_3$, pH 9·5 at a molar ratio of 1:100 at room temperature for 4 hr with continuous mixing by rotation. The mixture was then centrifuged and the supernatant was dialysed against phosphate-buffered saline pH 7·2. The degree of substitution was estimated spectrophotometrically, on the basis of the molar optical density of DNAP-lysine in 0·01 M NaOH at 415 nm. This latter compound was kindly synthesized and provided by Dr M. Löw (Chemical Works of Gedeon Richter, Budapest). Protein content was estimated by measuring the optical density at 280 nm. Since DNAP-lysine displayed an o.d. at 280 nm equalling 0·28 $E_{415\ nm}$, the measured $E_{280\ nm}$ of the protein conjugate was corrected accordingly. In this study $DNAP_{16}$-HSA and $DNAP_8$-IgG were used.

Lipopolysaccharide (LPS) from E. coli (kindly provided by Dr B. Ralovich, Department of Hygiene, Medical University of Pécs, Hungary) was treated with DNFA in 0·2M $NaHCO_3$ at ratio of 30:1 (w/w) for 2 hr at room temperature. The mixture was dialysed against PBS.

Immunization

Outbred 150–200 g, CFY rats of both sexes were injected with 100 μg of $DNAP_{16}$-HSA in Freund's complete adjuvant, intramuscularly. Four weeks later the same amount of antigen was given in incomplete adjuvant and the boosting was repeated after 8 days. The animals were exsanguinated on the eighth to tenth days following the last injection. DNAP-LPS (5 μg per animal) was injected intramuscularly in incomplete adjuvant. The injection was repeated 1 week later and the animals were bled out 4 weeks after the first injection.

Isolation of specific antibodies

$DNAP_8$-IgG or $DNAP_{16}$-HSA was coupled to CNBr-Sepharose 4B (Pharmacia, Uppsala) as suggested by the manufacturer. 20–25 ml of pooled immune serum was added to 15 ml (packed wet volume) antigen-immunoadsorbent and agitated by rotation at 4° for 24 hr. Antibodies were eluted by 0·35 M glycine-HCl buffer, pH 2·4 containing 0·1 M glucose. The eluate was dialysed against saline, then against 0·5 M boric acid to precipitate euglobulins. The precipitate was dissolved in 0·05 M acetic acid pH 5·5 containing 0·3 M NaCl and 0·02% NaN_3, and filtered through a Sephadex column as described previously (Medgyesi, Füst, Gergely & Bazin, 1978.) The supernatant was fractionated further by DEAE-cellulose chromatography (Medgyesi *et al.*, 1978). As an alternative procedure, the eluate was first resolved into IgM and IgG fractions by gel chromatography and the IgG fraction was applied onto SpA-Sepharose (Pharmacia) column. The dry adsorbent was swollen in water and washed with 0·01 M Na-phosphate buffer pH 8·0 containing 0·15 M NaCl and 0·05% NaN_3. The antibody fraction was applied after having been dialysed against the same buffer. The column was washed with the starting buffer (six times the bed volume). Elution was then started with 0·01 M Na-phosphate buffer pH 6·0 (in the same volume). The column was then washed with 0·15 M NaCl, 0·05%NaN_3 (one bed volume) and the elution continued by a sequence of 0·5 M, 1·0 M and 2·0 M $MgCl_2$. The column was stored in 0·15 M NaCl, 0·05% NaN_3 at 4°.

Analysis of IgG content

Antibody eluates and isolated fractions were analysed qualitatively by radial double immunodiffusion (Ouchterlony, 1958) and quantitatively single radial immunodiffusion (Mancini, Carbonara & Heremans, 1965). According to these measurements, the contamination of each subclass preparation with other immunoglobulin classes or subclasses was less then 5%.

Measurement of the antigen precipitating capacity and the avidity index of antibodies as well as the immune-complex solubilization assay were carried out as previously described (Rajnavölgyi *et al.*, 1978).

RESULTS

Isolation of antibodies

Anti-DNAP antibodies were efficiently eluted from antigen-immunoadsorbents at low pH, in contrast to anti-DNP antibodies (Eisen, Carsten & Belman, 1954). Antibodies from the DNA-IgG adsorbent did not react either with albumin or with IgG in passive haemagglutination but they reacted with DNAP-conjugated proteins (anti-hapten antibodies). Antibodies not fixed to DNAP-IgG could be bound to and eluted from the DNAP-HSA adsorbent. Eluted fractions agglutinated HSA-coated erythrocytes besides DNAP-HSA coated ones, however they precipitated more DNAP-HSA than non-conjugated HSA at equivalence (anti-hapten + carrier antibodies).

The recovery of serum antibodies in the anti-hapten

and in the anti-hapten + carrier fractions was estimated on the basis of precipitin equivalence. Antiserum pools were found to contain antibodies equivalent to 800–1700 μg $DNAP_{16}$-HSA per ml. The isolated anti-hapten antibody fraction precipitated 8%–15%, while the anti-hapten + carrier fraction precipitated 15%–25% of the antigen precipitated by the same volume of antiserum. The total recovery ranged from 24% to 32%.

Immunoglobulin isotype in the antibody fractions

The anti-hapten fraction isolated from each pool contained a small but sizeable amount of IgM. Three IgG subclasses, Gl, G2a and G2b were always present, while IgG2c was detected in some antiserum pools but not in the others. IgG2a was always the most abundant isotype.

In the anti-hapten + carrier fraction IgM and IgG2c were never detected. The other three IgG subclasses were contained by this fraction and relative amount of IgGl was higher than among the anti-hapten antibodies.

When DNAP-LPS was used for immunization the isolated antibody fraction was found to contain IgG2a only.

Isolation and properties of antibody subclasses

In the first series of experiments, antibody subclasses were separated by the general procedure proposed for rat immunoglobulin fractionation (Medgyesi *et al.*, 1978). The disadvantages of this technique are that IgG2c is recovered in a fraction containing some IgG2a and traces of IgG2b, furthermore IgG2b is mostly recovered as a minor component in a predominantly IgG2a containing fraction. These difficulties were alleviated by introducing affinity chromatography on Staphylococcus protein A. At pH 8·0 we have found almost no binding of IgG2a, therefore this subclass is recovered in the starting buffer. IgG2b can be eluted at pH 6·0 IgGl is eluted at lower concentrations of $MgCl_2$, while 2 M $MgCl_2$ is necessary to elute IgG2c. When IgM is also present among the antibodies, it is better to separate it from IgG by gel filtration before SpA affinity chromotography, since a portion of IgM was found to attach to the SpA adsorbent and this fraction was eluted by 2 M $MgCl_2$ only.

The avidity of antigen binding as reflected by the extent of the dependence of the antigen-binding capacity on the antigen concentration was determined by the method of Celada, Schmidt & Strom, 1969. At first, avidity of the antibodies in total serum and that of the antibodies eluted from antigen-immunoadsorbent have been compared. Only slight differences in the avidity indices were found (data not shown) indicating that no selective isolation of an antibody subpopulation occurred at affinity chromatography. When the avidity of isolated antibody subclasses was

Table 1. Properties of rat anti-DNAP antibody fractions

	Avidity index	Maximal C† consumption (%)
Total serum	0·57	77
Anti-hapten antibody		
IgM	0·70	10
IgGl	0·70	20
IgG2a	0·25	45
IgG2b	0·37	52
IgG2c	0·35	15
IgG2c + IgG2a	0·66	50
IgG2a + IgG2b	0·40	80
IgG2a*	0·36	18
Anti-hapten + carrier antibody		
IgGl	0·75	10
IgG2a + IgGl + IgG2b	0·46	—

* From antiserum produced by immunization by DNAP-LPS.

† C, consumption from rat serum by 30 μg antibody plus an optimal dose of $DNAP_{16}$HSA antigen.

measured. IgG2a-antibody fractions appeared to be less avid than those containing the other IgG subclasses or IgM. All IgG subclasses and IgM were found to precipitate the antigen while IgG2a had a low precipitating capacity (Table 1).

Interaction of antigen–antibody complexes with the complement system

Rat serum was used as a complement source to estimate the complement activating capacity of antigen–antibody complexes. The complement activating capacity of complexes containing unseparated antibodies of defined antibody isotype(s) was estimated by measuring the consumption of total complement activity from homologous serum. The same amounts of antibody in total serum and in the eluate from antigen-immunoadsorbent in complexed form induced comparable extent of complement consumption (data not shown). Furthermore, maximal consumption was found at the same antigen/antibody ratios with both kind of antibodies. All the IgG subclasses possessed measurable complement-consuming activity (Table 1, Fig.1). The greatest capacity to activate complement was found in fractions containing IgG2b and IgG2a. Complexes with IgGl-type antibodies were usually less efficient, those containing IgG2c-type antibodies were active only weakly.

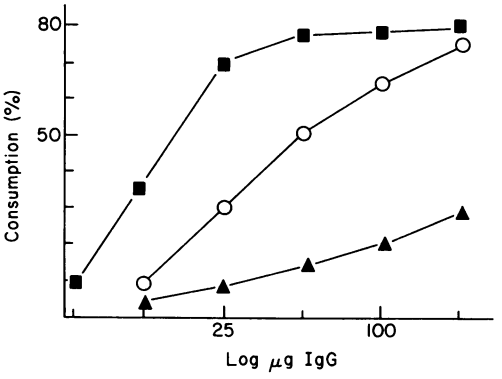

Figure 1. Total complement consumption in rat serum by immune complexes containing $DNAP_{16}$-HSA antigen and purified anti-DNAP antibody preparations of different subclasses: (■——■) IgG2b; (○——○) IgG2a; (▲——▲) IgGl.

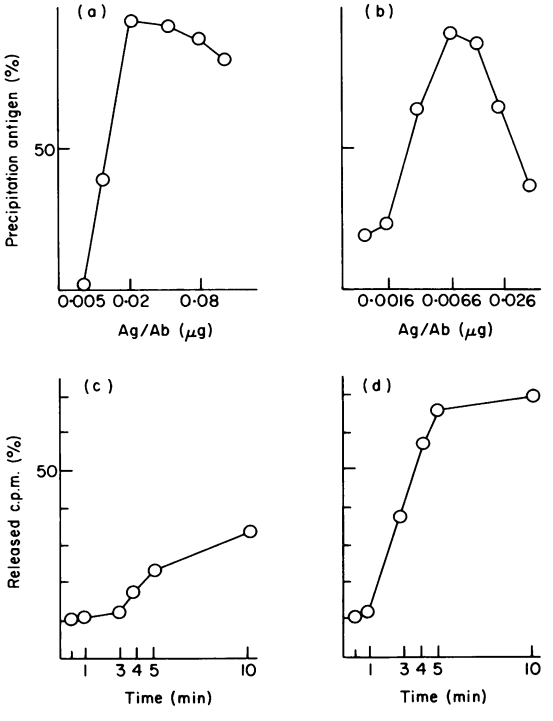

Figure 2. Precipitation curve (a,b) and complement dependent dissolution (c,d) of immune complexes containing $DNAP_{16}$-HSA antigen and anti-DNAP antibody preparation of IgG2c + IgG2a subclasses (a,) and of IgG2a + IgG2b subclasses (b,d).

The complement-dependent dissolution of precipitated complexes was also studied. Precipitates formed at equivalence were compared, and in most of the instances a rapid dissolution was observed, reaching the plateau level in 5–10 min. The only remarkable exception was the precipitate containing predominantly IgG2c antibodies displaying a much lower rate and extent of dissolution (Fig. 2). A similarly slow rate of solubilization was found with precipitates formed of whole antiserum and a non-fractionated antibody fraction eluted from affinity chromatography column, respectively.

DISCUSSION

In this study we have examined the properties of rat IgG subclasses and of IgG in the form of antigen–antibody complexes. The antibody fractions investigated were isolated from a polyclonal antibody population by use of a suitable combination of separation techniques. Affinity chromatography on SpA-Sepharose was proved to be the most efficient way of IgG subclass separation. Isolation of mouse (Ey, Prowse & Jenkin, 1978, Chalon, Milne & Vaerman, 1979) IgG subclasses on the same adsorbent has already been reported. In agreement with previous studies (Medgyesi *et al.*, 1978) we have found the quantitatively dominant subclass (IgG2a) to pass through the adsorbent without being retained while the most firmly fixed subclass was IgG2c.

It seems most probable that no highly selected subpopulations of antibody isotypes were recovered and therefore the results of this study may represent the subclasses as a whole: (a) no selective isolation of the antibodies occurred at the antigen-immunoadsorption since the eluted fractions had the same avidity and complement consuming capacity as the antibodies in the whole serum; (b) in the complement activating capacity or avidity no significant differences were observed between antibody isotype preparations isolated either by DEAE-cellulose chromatography or by affinity chromatography on SpA-Sepharose, indicating again that the procedures applied in this study did not result in separation of highly selected antibody populations.

Examining the composition of antisera and the antigen-binding properties of the isolated immunoglobulin subclasses the most remarkable finding was the consequently low avidity of IgG2a antibodies. Regarding that only this subclass was found in the antibodies produced when DNAP-LPS was used for immunization we assume a difference in the dependence on T-cell co-operation of the production of different antibody subclasses. The sequence of cellular events leading to IgG2a-antibody production may be independent of T cells at certain stages and as a consequence, this subclass of antibody may mature slower in terms of avidity than other subclasses.

When testing the complement activating capacity of antigen–antibody complexes we used homologous serum as a complement source. In a separate study we have found evidence for differences in the complement activating capacity of the same aggregated immunoglobulins when complement consumption from homologous serum and from heterologous sera was compared (Füst, Medgyesi, Bazin & Gergely, 1980). The low complement activating capacity of IgM is concordant with a recent report of Klaus (1979) on mouse antigen–antibody complexes. The relatively high complement activating capacity of IgG2a-containing complexes is remarkable. These low avidity antibodies may activate and bind complement components, thus IgG2a-containing complexes can induce complement-dependent effects. According to preliminary (unpublished) results from this laboratory, the presentation of antigen with IgG2a antibodies enhances the secondary antibody response in comparison with the results of immunization with antigen only. Such an effect is thought to be the consequence of the complement-mediated attachment of the complex to dendritic cell membranes (Klaus, 1978). Complexes containing IgG1-type antibody displayed only a low capacity to activate homologous complement. In a previous study on rabbit IgG antibodies (Rajnavölgyi *et al.*, 1978) a general tendency of higher avidity fractions to be more efficient in complement activation was revealed. The behaviour of IgG1-type antibodies in this study is not consistent with the correlation mentioned above, since antibodies of this subclass were usually highly avid while their complexes were relatively inefficient in activating complement. Intrinsic structural properties IgG1 may be responsible for this deviation. In rabbits IgG subclasses have not been established thus the antibody populations compared in our earlier study might not have subclass heterogeneity.

We intend to note that IgG2c-containing complexes were able to consume homologous complement. Based on the lack of consumption of human complement by aggregated monoclonal IgG2c proteins this subclass was thought to be unable to activate complement (Medgyesi *et al.*, 1978). Aggregated IgG2c

proteins however were found to consume complement through the classical but not through the alternative pathway (Füst *et al.*, 1980). The rate and extent of complement-dependent solubilization of insoluble complexes of IgG2c antibodies was lower in comparison to complexes of the other IgG subclasses. The inefficiency of IgG2c immunoglobulins to activate the alternative pathway may explain the low rate of solubilization since the amplification of C2 conversion by the alternative mechanisms is thought to be decisive in the CRA process (Takahashi *et al.*, 1976).

ACKNOWLEDGMENTS

It is a pleasure to acknowledge the expert technical assistance of Ms Kinga Ambrus, Ms Katalin Dékány and Ms Biri Klenóczy.

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