STUDIES OF THE ACTION OF SOME ANTI-INFLAMMATORY DRUGS ON COMPLEMENT MEDIATED IMMUNE HAEMOLYSIS

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1 The effects of various anti-inflammatory and non-anti-inflammatory drugs on complement mediated haemolysis have been studied. Drugs which were significantly protein bound were found to inhibit this form of immune lysis, but only at greater concentrations than achieved therapeutically.
2 Removal of the drugs by prolonged dialysis resulted in restoration of complement activity with the exceptions of phenylbutazone and warfarin sodium.
3 Reconstitution experiments indicated that C2 and some of the later components especially C7 were affected by the drugs.
4 Intra-articular injections of prednisolone (100 mg) in patients with rheumatoid arthritis, failed to produce significant changes in the synovial fluid complement system.
5 None of the drugs affected the binding of antibody to antigen, or the ability of sensitized sheep cells to fix complement.

Introduction

The complement system through the action of its nine components and various cleavage products plays a major role in the inflammatory response. Chronic inflammation is a characteristic feature of the chronic arthritis diseases, and there is now convincing evidence that the complement system is activated in both seropositive (Hedberg, 1963, 1964, 1967; Pekin & Zvaifler, 1964; Postiropoulos, Austen & Bloch, 1965; Barnett, Bienenstock & Bloch, 1966; Peltier, Coste & Delbarre, 1966; Vaughan, Barnett, Sobel & Jacox, 1968; Zvaifler, 1969; Rudy & Austen, 1970; Hedberg, Lundh & Laurell, 1970; Gilgore, Bolishu, Duter & Podut, 1971; Rudy, Everson, Schur & Austen, 1971; Rudy, Muller-Eberhard & Austen, 1971; Britton & Schur, 1971; Ward & Zvaifler, 1971; Versey, Hobbs & Holt, 1973; and seronegative (Whaley, Canesi, Moseley, Morrow, Sturrock, Mitchell & Dick, 1974) arthritides. As there is at present no cure for these disorders, treatment is simply symptomatic. The most effective drugs are those with anti-inflammatory properties. The present study was undertaken to investigate the possible actions of certain anti-inflammatory drugs on the complement system in vitro. Several drugs without anti-inflammatory properties served as controls.

Methods

Drugs

Prednisolone sodium phosphate, sodium salicylate, chloroquine diphosphate and phenylbutazone were the anti-inflammatory drugs studied. The control drugs, selected because of their lack of significant anti-inflammatory properties, were warfarin sodium, cloxacillin sodium and isoniazid. Solutions of the drugs were made up on the day of testing, by dissolving the drug in complement fixation diluent to a concentration of 20 mg/ml. The only exception to this was phenylbutazone which was made up by dissolving 200 mg in 0.5 ml N sodium hydroxide solution and then making up

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to a final concentration of 5 mg/ml by dilution in complement fixation diluent.

Complement fixation diluent (Oxoid Ltd) was made up by dissolving one tablet in distilled water (100 ml).

**Complement studies**

Normal human serum served as a source of complement. Serum was separated from whole blood from a healthy volunteer and stored at −30°C in aliquots for up to one week. Aliquots were frozen and thawed once only. Each week fresh serum was obtained.

Total haemolytic complement was measured by the method of Kent & Fife (1963). Serum was initially diluted 1 in 15 with complement fixation diluent, mixed with an equal volume of drug solution, and incubated at 37°C for 1 h prior to titration. The number of 50% haemolytic units of complement (CH50 units/ml was calculated using von Krogh's equation (von Krogh, 1916) and then calculated as a percentage of the number of CH50 units present in the control titration. (Control 1/15 serum dilution further diluted with an equal volume of diluent without drug.) Drugs were initially screened at a final concentration of 10 mg/ml and then by doubling dilutions. The pH of the drug/serum mixtures was 7.2.

In a second series of experiments, drug solutions, as prepared previously, were incubated with equal volumes of undiluted serum for 1 h at 37°C, then diluted in complement fixation diluent to a final 1/30 dilution, and finally titrated as above.

Apart from total haemolytic complement concentrations, serum concentrations of certain individual components were measured after the incubation of neat serum with an equal volume of drug solution for 1 h at 37°C. This was performed to see whether destruction of any complement components had occurred. Examination of the sera for cleavage of C3 or GBG (C3-proactivator) was performed to test for activation of the complement system.

Serum concentrations of C1q, C3 and GBG were measured by radial immunodiffusion using monospecific antisera prepared as follows: C1q – Immunization of rabbits with latex particles agglutinated in fresh normal human serum (Thunold, Abeyounis & Milgrom, 1970) or with purified C1q (Agnello, Winchester & Kunkel, 1970; Yonemasu & Stroud, 1971). Sera were absorbed with lyophilized heat-inactivated human serum if required. C3 – Rabbits were immunized with zymosan previously incubated in human serum (Mardiney & Muller-Eberhard, 1965) or with alexinated bacteria (Thompson, 1972).

Absorption with low C3 containing sera was sometimes required. GBG – Rabbits were immunized with immune precipitates prepared by immunoelectrophoresis (Goudie, Horne & Wilkinson, 1966). C7 – Concentrations were measured in agarose plates using the reactive lysis procedure (Thompson & Lachmann, 1970). The plates contained unsensitized sheep erythrocytes, activated C56 and 0.01 M EDTA. C3 conversion was measured by crossed antibody electrophoresis (Laurell, 1965) and GBG conversion to glycine-rich-gamma-glycoprotein (GGG) was detected by immunoelectrophoresis. Both tests were performed in 1% agarose containing 0.01 M EDTA.

**Dialysis Experiments**

These were undertaken to investigate whether the anti-complementary effect of the drugs studied could be removed by dialysing the drug out of the drug/serum mixture. Drug/serum mixtures were prepared as previously described, using both 1/15 diluted serum and undiluted serum. The mixture was placed in Visking dialysis tubing and dialysed against 100 volumes of complement fixation diluent. The exact volumes were 10 ml of 1/15 serum/drug mixture against 1 litre of diluent, and 2 ml undiluted serum/drug mixture against 200 ml. The concentrations of both drug and haemolytic complement were measured at 3, 24, 48 and 72 hours. The dialysates were changed for fresh diluent at 24 and 48 hours. Controls without drugs were included in all experiments.

Drug concentrations were determined by spectrophotometric analysis of the dialysis sac contents at the wavelengths of maximum absorption as follows: chloroquine, 343 nm; isoniazid, 261 nm; phenylbutazone, 263 nm; prednisolone, 246 nm; sodium salicylate, 295 nm; warfarin sodium, 307 nm. Appropriate standards and blank solutions were used in each case.

**Effects of drugs on antigen-antibody binding, and complement fixation by immune complexes**

Effects of drugs on the binding of antibody to sheep erythrocytes, and the ability of sensitized sheep cells to fix complements were also investigated as follows:

(i) Washed sheep cells were incubated with drug solutions (10 mg/ml) at 37°C for 1 h, followed by three washes in diluent. The concentration of cells was then adjusted to 1 x 10^9 cells/ml and sensitized with antibody as for the standard preparation of sensitized cells for the complement titration procedure.

(ii) Antibody to sheep red cells optimally diluted
was incubated for 1 h at 37°C at a drug concentration of 10 mg/ml, and then incubated with the standardized sheep red cell suspension as for the preparation of sensitized cells for the complement titration. The sensitized cells were then washed thrice in diluent and then restandardized spectrophotometrically.

(iii) Sensitized cells were incubated for 1 h at 37°C at drug concentrations of 10 mg/ml and then washed thrice in diluent and restandardized spectrophotometrically. The different preparations of sensitized cells were then used in titrations of the same batch of normal human complement.

The effect of the drugs studied on the total amount of antibody bound to red cells was measured by a radioactive anti-globulin test (Neilsen, Parratt & White, 1973). Fresh human red cells, Group A, D positive, were washed five times in diluent, and finally adjusted to a 50% suspension in diluent. Volumes of the cell suspension (100 μl) were added to a series of test tubes, followed by serum (100 μl) containing either high titre anti-A-antibody (IgM) or high titre anti-D antibody (IgG). The cells were then incubated at room temperature for 1 h, washed thrice in diluent, and then reincubated with 125I-labelled globulin having either anti-IgM or anti-IgG activity. The amount of labelled antibody needed to be in excess was previously determined by titration.

The cells were again incubated at room temperature for 1 h, washed thrice in diluent, and the tubes counted in an automatic well gamma counter (Nuclear Chicago). The counts per tube are directly proportional to the amount of antibody bound to the red cells. The incubation of red cells, with serum was done in the presence and absence of drugs, the final drug concentration being 10 mg/ml, except phenylbutazone which was at 5 mg/ml.

**Effect of intra-articular prednisolone on synovial fluid total complement**

As the experiments progressed, it became apparent that the drugs studied produced inhibition of complement mediated haemolysis, so it was decided to observe the effects of prednisolone (100 mg) injected intra-articularly into the knee joint in order to achieve a high drug concentration locally. Ten patients with classical rheumatoid arthritis (Ropes, Bennett, Cobb, Jacox & Jessar, 1958) having large synovial effusions consented to be studied. Prior to the injection of prednisolone, synovial fluid (10 ml) was aspirated, and divided equally into two aliquots, one of which was allowed to clot, and the other anticoagulated with EDTA. Specimens were kept at 4°C and centrifuged within 2 h of aspiration and then frozen at −70°C until use. Six knees were injected with prednisolone (100 mg) and four with sterile saline. Synovial fluid samples were taken again after 30 min, 60 min and 24 hours. Estimations on the synovial fluid specimen included total haemolytic complement, C1q, C4, C3, C7 and GBG concentrations, and C3 and GBG conversions.

**Reconstitution experiments**

Attempts to identify the site(s) of the inhibitory actions of the drugs studied were made by attempting to restore haemolysis using heat inactivated serum (depleted of C1 and C2), ammonia-treated serum (depleted of C4), zymosan treated serum (depleted of C3 and the terminal C5-9 components), the acid euglobulin component of serum (depleted of C1 and C3) and the supernatant following separation of the acid euglobulin fraction (depleted of C2 and C4). None of the preparations had haemolytic activity, neither were they anti-complementary. All the components were titrated against each other to determine the required volume to achieve complete lysis (Mayer, 1961). Only undiluted serum/drug mixtures were tested. In order to prevent the drug impairing the activity of the reagents, the concentrations of drug used was insufficient to produce complete inhibition of haemolysis. For prednisolone, sodium salicylate and cloxacillin, a final concentration of 10 mg/ml was used, whereas for warfarin sodium it was 5 mg/ml and for phenylbutazone, it was 2.5 mg/ml.

**Results**

**Effects of drug concentration on complement mediated haemolysis**

The results are shown in Figure 1. All the drugs studied were anti-complementary to variable extents, when incubated with serum diluted 1/15. The effect of isoniazid was only marginal even at high concentrations, whereas warfarin sodium and phenylbutazone were markedly anticomplementary, and even showed over 10% inhibition at a concentration of 1.25 mg/ml. Sodium salicylate and prednisolone were almost as strongly anti-complementary as warfarin and phenylbutazone, whereas cloxacillin and chloroquine were inter-
mediated inhibitors of complement mediated haemolysis, the degree of inhibition falling between that observed with prednisolone and salicylate and that with isoniazid. When undiluted serum was used as the source of complement, less inhibition of haemolysis occurred for the same drug concentrations. Isoniazid and chloroquine produced no inhibition of lysis, and markedly reduced inhibition occurred with sodium salicylate, prednisolone and cloxacillin. The degree of inhibition with warfarin sodium and phenylbutazone was only slightly less when undiluted serum was used.

**Effect of dialysis on drug-induced inhibition of complement mediated haemolysis**

The results are shown in Figure 2. The rates at which the drugs were dialysed out of the sacs were similar when both undiluted and diluted serum were used. We have therefore presented the results for diluted serum only. Sodium salicylate, chloroquine and isoniazid were to a large extent dialysed out of the sac within 3 h, and this paralleled a return to normal of the haemolytic complement concentration. Prednisolone, phenylbutazone and warfarin sodium were removed more slowly without any marked difference between them. With prednisolone in undiluted serum, complete haemolysis was restored by 3 h, whereas in dilute serum it took 48 h for restoration. With phenylbutazone, even though only trace quantities of drug remained after 48 h, only 20-25% haemolysis was present. Likewise, the complete inhibition of complement mediated haemolysis induced by warfarin sodium, persisted despite undetectable drug concentrations.

Cloxacillin was not studied during prolonged dialysis due to its instability in solution.

**Effect of drugs on antigen-antibody binding and complement fixation by immune complexes**

No observable effect of drugs on the binding of antibody to sheep red cells, by their pre-
incubation with any drug, was demonstrable by the techniques used. In addition, the incubation of sensitized sheep cells with any drugs did not inhibit complement fixation. Chloroquine could not be investigated in these experiments due to its direct lytic effect on red cells. The results of the antiglobulin tests showed that none of the drugs studied was shown to impair the binding of either IgG or IgM antibody at the concentrations studied.

Reconstitution experiments

The results are shown in Figure 3. Complement mediated lysis was not only restored, but enhanced by the addition of the acid euglobulin supernatant (depleted of C1 and C3) and ammonia-treated serum (depleted of C4). Heat-inactivated serum (depleted of C1 and C2) zymosan treated serum (depleted of C3 and C5-9), and the acid euglobulin precipitate (depleted of C2 and C4) failed to restore complete lysis.

Complement component concentrations

Concentrations of C1q, C4, C3, C7 and GBG in the drug/undiluted serum mixtures were normal, although the development of the zone of reactive haemolysis in the C7 plates was delayed in the drug-treated sera (2 or 3 days) compared with controls (1 day). Conversion of C3 and GBG was not noted in any of the drug/undiluted serum mixtures.

Action of intra-articular prednisolone on the complement system

No significant changes in total haemolytic complement, C1q, C4, C3, C7 or GBG concentrations, on the degrees of C3 and GBG conversion were found in either the prednisolone treated patients or the controls.

Discussion

All the drugs inhibited the complement system by varying degrees, the extent of inhibition being dependent upon drug concentrations (Figure 1). The ability of the drugs to inhibit the system appears to be related to their protein-binding. Thus, isoniazid which is not protein-bound (Lass, Tope & Wunderlich, 1955) inhibits the system only to a limited extent. Chloroquine, a drug which is only moderately bound (Parker & Irvine, 1952) was intermediate in its inhibitory effect. Warfarin sodium and phenylbutazone which are extensively protein-bound (Weiner, Shapiro, Axelrod, Cooper & Brodie, 1950; Burns, Rose, Chenkin, Goldman, Schulbert & Brodie, 1953) produce complete inhibition of the system. Salicylate and prednisolone which are also regarded (Lester, Lolli & Greenberg, 1946; Cope, 1972) as strongly protein-bound inhibit the system to a slightly lesser extent than warfarin sodium and phenylbutazone.

Both anti-inflammatory and non-anti-inflammatory drugs (cloxacillin and warfarin sodium) were able to inhibit the complement system, showing that inhibition of complement mediated immune haemolysis is not a property peculiar to the anti-inflammatory drugs.

When diluted serum rather than undiluted serum was used as the complement source, greater inhibition of haemolysis was observed. This suggests that the drug is initially bound to conventional drug binding sites (e.g. albumin), and
only when these are saturated is there an overflow on to other secondary binding proteins, which include certain complement binding components. The inhibition produced by sodium salicylate, prednisolone, chloroquine and isoniazid was reversible as shown by the results of the dialysis experiments. The time for the return of 100% haemolysis being related to the rate of removal of the drug. However, warfarin sodium and phenylbutazone treated serum still showed marked inhibition of lysis even when there was virtually no drug detectable by the spectrophotometric method used. This could be explained in three ways:

(1) The amount of drug bound to the complement proteins in sufficient concentration to be inhibited is so small as to be undetectable by the techniques used, and warfarin sodium and phenylbutazone are bound more strongly to these proteins than to the conventional binding sites. While the spectrophotometric methods used are not the most sensitive methods available, they were sufficiently sensitive (limit of sensitivity about 0.05 mg/ml) to determine the concentrations of drugs which were previously shown to be insufficient to inhibit haemolysis (Figure 1).

(2) The drugs are all reversibly bound to the complement proteins, but warfarin sodium and phenylbutazone in some way produce irreversible changes in the proteins so as to abolish their haemolytic properties.

(3) Warfarin sodium and phenylbutazone may activate the complement system with subsequent depletion of certain components which would reduce the haemolytic complement titre. This is unlikely as the concentrations of C1q, C3, C4, C7 and GBG were unaltered after incubation, and conversion of C3 or GBG did not occur.

The results of the reconstitution experiments were the same for all the drugs studied, suggesting that they were all acting at the same sites in the complement cascade. With the exception of the failure of zymosan treated serum to restore lysis, the results clearly show that C2 is the component affected. C2 is the limiting early component (Mayer, 1961) and the excess of C2 in the ammonia-treated serum and the acid euglobulin supernatant will account for the excessive lysis observed following the addition of these reagents. The failure of zymosan treated serum to restore lysis, and the restoration achieved by the acid euglobulin supernatant, both of which are depleted of C3, appear to be contradictory. One explanation is that zymosan treated serum is depleted not only of C3, but also the later components (C5-9), and that certain of these components may be found in both the acid euglobulin precipitate and supernatant. C7 for instance is known to be distributed in both the acid euglobulin precipitate and supernatant (Thompson & Rowe, 1968), and the delayed appearance of zones reactive lysis on the C7 plates suggests that the drugs act on C7. Thus only a reagent containing both C2 and C7, and possibly other later acting components, could restore lysis.

The high concentration of these drugs necessary to achieve inhibition of complement mediated immune haemolysis, and the failure of intra-articular prednisolone to produce any significant effects on the complement system, suggest that the anti-inflammatory drugs studied do not act by inhibiting the complement cascade, although perhaps their metabolites formed in vivo, may do so.

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References


Gligore, V., Bolishu, H.D., Duter, H. & Podut,


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