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HYPOCHLORITE-INDUCED ALTERATIONS TO CANINE SERUM COMPLEMENT

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SUMMARY

Changes in the concentration of the components of complement produced by NaOCl both *in vitro* and *in vivo* are recorded. C1, C4 and C7 are particularly sensitive to this oxidizing agent, although all components decrease at high concentrations of NaOCl. Following oxidation, complement components return rapidly to normal. Data are presented to indicate that part of this repair mechanism is due to the action of reducing agents such as ascorbic acid and part is due to the synthesis of the individual components. The unique sensitivity of complement components to oxidation make this treatment of potential value in suppressing the inflammatory response.

INTRODUCTION

In previous reports it was shown that sodium hypochlorite (NaOCl) administered intravenously or across the membrane of a Kiil dialyser is an effective decomplementing agent and could significantly prolong survival of porcine kidney xenografts in dogs (Bier *et al.*, 1971, 1973). It was established that depression of complement titres (C'H50) was dose-dependent and that survival of xenografts was prolonged equally in animals whose C'H50 levels were reduced to 50–70% as in animals whose C'H50 levels were reduced to zero (Bier *et al.*, 1973). Of clinical significance, NaOCl selectively inactivated complement and coagulation proteins, had an immediate action, and was devoid of discernable systemic effects. The major limitation in the use of NaOCl as a means of prolonging xenograft survival was the necessity of using repeated treatments in order to maintain a hypocomplementary state; complement activity was shown to return rapidly to normal as soon as the administration of the oxidizing agent was stooped (Bier *et al.*, 1973). It was suggested that ascorbic acid, which is synthesized *in vivo*, and is present in significant quantities in dog serum, plays an important role in the reactivation of oxidized complement. No information on the changes in the individual components of complement were presented.

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The present report investigates the effects of NaOCl administered *in vivo* or *in vitro* on the individual components of dog serum complement. In addition, the effects of ascorbic acid on the complement components of oxidized serum are studied.

MATERIALS AND METHODS

In vivo studies

Sodium hypochlorite, 0·08 N, was prepared by diluting 60 ml of commercial NaOCl (The Chlorox Corporation, Oakland, California) into 1000 ml of sterile saline, and adjusting the pH to 7·4 with hydrochloric acid. Its normality was checked iodometrically using standard procedures (Bier *et al.*, 1971; Horowitz, 1955). A fresh solution was prepared for each administration since the solution was not stable.

For the continuous separation of blood into plasma and packed cells, a Celltrifuge (American Instrument Company, Silver Spring, Maryland) was used. The plasma flow was adjusted to 1·5 ml/min/lb body weight of the treated dog(s). Packed cell flow maintained as necessary to clear the rotor. NaOCl was infused into the plasma line emerging from the rotor, and a holding reservoir was inserted into the line with sufficient capacity to provide about 2 min residence time before mixing it with the packed cells. Mongrel dogs weighing in excess of 30 kg were used. To provide access to the blood supply, a carotoid–jugular shunt was established in the neck 1 day before treatment (Bier *et al.*, 1973).

Six equal doses of NaOCl containing 4 mmoles each were administered. The first dose was given intravenously. The remaining five doses were mixed with plasma, using the Celltrifuge. Blood samples were taken at the beginning of the experiment (control), midway through the chlorination procedure (sample 1), immediately following the chlorination (sample 2), 4 hr after the last administration of NaOCl (sample 3), and 24 hr after the last chlorination (sample 4). All serum samples were prepared as rapidly as possible, put into small aliquots and frozen at −70°C until assayed.

In vitro studies

Physiologic saline was used as a diluent for NaOCl, or ascorbic acid (Mallinckrodt Chemical Works, St Louis, Missouri) to the desired concentrations. Gelatin veronal buffer (GVB), pH 7·35, was prepared daily (Boyer & Wyde, in preparation). Equal volumes of this buffer were mixed with 5% glucose (Gl–GVB) and used as a diluent for all C'H50 and complement component assays. Serum for use in *in vitro* studies was prepared from the clotted blood of normal dogs and stored at −70°C until needed. On the day of an experiment, seven equal 1·6 ml portions of serum were prepared, allowed to reach 37°C, and NaOCl, ascorbic acid or saline added to each. All mixtures were incubated for a total of 30 min at 37°C. At the end of this period the tubes were transferred to an ice bath. All samples were portioned into 0·1 ml aliquots and stored at −70°C until assayed for complement activity. Samples of the sera obtained during the *in vivo* and *in vitro* experiments were assayed for whole complement and component activity using haemolytic assays. Each sample to be tested was quickly thawed on the day of an assay and maintained in an ice bath.

Complement assays

Total complement (C'H50) and the nine individual components of the classic pathway were measured as described in detail elsewhere (Boyer & Wyde, in preparation). In brief: C1,

C2, C3, C5, C8 and C9 were assayed by using cellular intermediates modified after the methods of Rapp & Borsos (1970). C4, C6 and C7 were assayed by dilution in the whole, diluted serum of guinea-pig (Gaither & Frank, 1973), rabbit (Rother *et al.*, 1966), and man (Boyer *et al.*, in preparation) deficient in each of these, respectively. Titres varied considerably from day to day. Therefore, a correction was made by reference to a standard serum tested each day and results were expressed as percentage change in titre. By this method all results were highly reproducible.

RESULTS

Effects of NaOCl and ascorbic acid on canine serum complement in vitro

Table 1 shows the effect of NaOCl at two dosage levels on serum complement components in a representative experiment. Equimolar ascorbic acid was employed to effect partial restoration in each case. As was reported previously (Bier *et al.*, 1971), marked diminution

TABLE 1. Effects of NaOCl and ascorbic acid on canine serum complement *in vitro*

Reagents added*		Complement assay as percentage of control									
1st incubation (NaOCl)	2nd incubation (Asc Ac)	C'H50	C'1	C'2	C'3	C'4	C'5	C'6	C'7	C'8	C'9
0·007 M	Buffer	0	7	30	2	3	3	2	1	2	3
0·007 M	0·007 M	7	3	26	79	5	10	47	8	39	81
Buffer	0·007 M	100	96	119	74	65	67	76	73	56	67
0·0014 M	Buffer	13	9	70	76	9	56	87	14	58	72
0·0014 M	0·0014 M	33	12	83	90	61	98	69	14	67	116
Buffer	0·0014 M	124	85	92	115	91	63	74	88	67	67

* After an initial incubation for 5 min at 37°C with the first reagent, the second reagent was added and the mixture incubated an additional 25 min. Controls had buffer added in both instances.

TABLE 2. The effects of NaOCl administered *in vivo* on canine complement

Sample*	Component activity†									
	C'H50	C'1	C'2	C'3	C'4	C'5	C'6	C'7	C'8	C'9
Control (u/ml)	95	24,000	2650	19,000	23,000	3000	2100	4900	360,000	215,000
1	56	18	25	31	23	35	44	28	58	38
2	23	5	20	10	15	35	34	1	34	28
3	45	25	25	26	59	51	42	7	68	90
4	110	85	90	100	91	116	59	70	100	100

Twenty-four mmoles of NaOCl were infused into a 33 kg dog.

* Samples were collected: (1) at the midpoint of infusion; (2) at the end of infusion; (3) 4 hr later; (4) 24 hr later.

† Control values are reported in haemolytic units per millilitre; other values as a percentage of the control.

of C'H50 was obtained with both 0·007 M and 0·0014 M NaOCl. At the high dosage, all components, but C2, were markedly depressed. At 0·0014 M NaOCl, however, only C1, C4 and C7 were greatly decreased. Restoration by ascorbic acid was pronounced for C3, C6, C8 and C9 at the higher concentrations and additionally for C4 at lower concentrations. C1, C2, C6 and C7 were relatively little affected by the ascorbic acid. Ascorbic acid alone caused slight decreases in component titres except for C3 at the lower dose, and C2 at the higher, which were augmented by ascorbic acid.

Effects of NaOCl on complement in vivo

Table 2 and Fig. 1 show the results of a representative experiment. Control values in units/ml are reported in the first line of Table 1. The remaining values for canine serum in the Table and in Fig. 1 are reported as a percentage of the control.

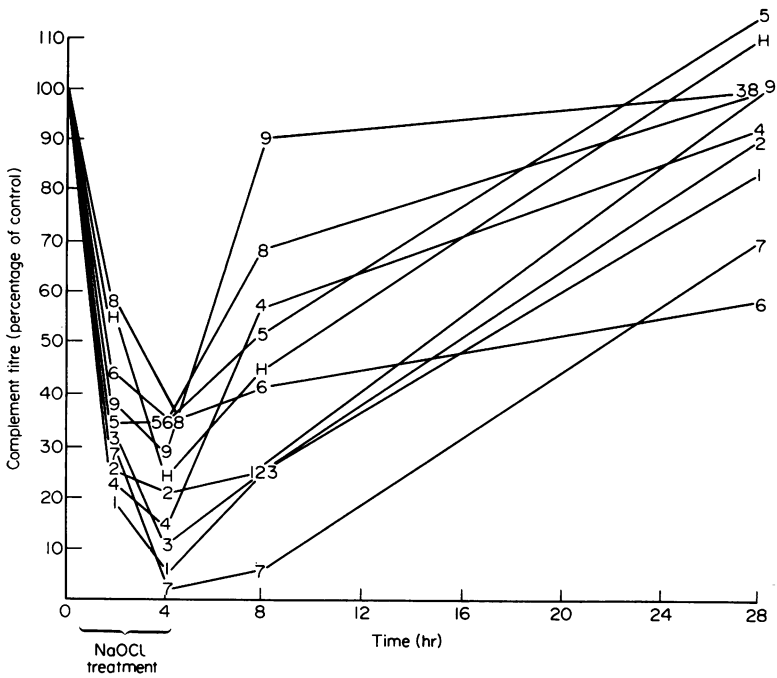

FIG. 1. Response of serum complement components (C'H50 and C'1 – C'9) to NaOCl infusion, measured as complement titre (as a percentage of the control).

DISCUSSION

These experiments confirm earlier work showing falls in total serum complement following infusion of NaOCl *in vivo* or by direct mixture of the oxidant with serum *in vitro*. In particular, the rapid fall in C'H50 following NaOCl infusion and the return to normal within 24 hr is again seen. They extend the earlier data by adding analyses of the nine specific components of the classic complement pathway. The assays employed were not entirely suitable because incompatibilities between human and dog, and between guinea-pig and dog C4 and C2 have

been described (Gigli & Austen, 1971; Sargent & Austen, 1970). However, the changes found were remarkably consistent and reproducible and it is assumed that they reasonably reflect the true events.

All components of the classical pathway were susceptible to oxidative destruction. *In vitro* and *in vivo* the first, fourth, and seventh components were particularly sensitive. However, there were also certain inconsistencies between *in vitro* and *in vivo* conditions: C3 was fairly insensitive *in vitro* and sensitive *in vivo*; C5 was one of the least sensitive *in vivo* and one of the more sensitive *in vitro*.

The decrease in C1 to 5% and C7 to 2% of control level by the end of the chlorination while the total complement (C'H50) decreased only to 24% may explain previous data indicating that xenograft survival was prolonged as well by reduction of C'H50 to 50% as it was to zero (Bier *et al.*, 1971). It would seem likely that the extreme decrease of C1 might prevent activation of the classical complement pathway and, thus, the rejection response. Similarly, low C7 in spite of sufficient other components could stop the complement cascade from both classical and alternative pathways from progressing to cytotoxicity.

Repair of components following oxidation was particularly marked for C4, C5 and C9 *in vitro* by the addition of ascorbic acid. It is of interest that these three components also appear to recover most rapidly after the end of the infusion *in vivo*. This is consistent with the earlier suggestion that it is circulating reducing agents, especially serum ascorbic acid, which may reverse the effects of the oxidants *in vivo* (Bier *et al.*, 1971). *In vitro* reversal of complement inactivation by oxidants using reducing agents was originally shown by Ecker *et al.* (1937).

The rise in components following NaOCl infusion could also be explained by shifts of extravascular pools or by synthesis, but there is nothing in the present data to help evaluate the relative importance of either. C9, a relatively small molecule, might enjoy an advantage through more rapid diffusion from extravascular pools. However, C4, with three times the size (Cooper, Polley & Müller-Eberhard, 1971), reappears just as rapidly. Perhaps the best clue for this rapid recovery comes from earlier data (Bier *et al.*, 1971): even after 12 days of intermittent treatments of NaOCl, the rate of C'H50 rise following cessation of therapy was undiminished. Extravascular pools should have been depleted during this period, and only synthesis could explain the rapid rise. Through this interpretation, C4 and C9 are the most rapidly and C6 and C7 the least rapidly synthesized.

Comparison of the sensitivity of complement components of the dog with those of man (Boyer & Wyde, in preparation) are in order: C1 is particularly sensitive and C3 moderately sensitive in each system. C7 and C4 are very sensitive in dog complement, but resistant in human. C2 is very sensitive in man, but not in dogs. Since susceptibility to oxidative destruction is thought to be due to the presence of sulphhydryl or disulphide groups on the protein molecule (Ecker *et al.*, 1937; Bier *et al.*, 1971), it would seem reasonable to conclude that, in the dog C1, C4 and C7 and in man C1, C2 and C3 are rich in these sulphur groups. In this context, it is interesting that at concentrations of NaOCl which destroy all complement components totally, serum SGOT, alkaline phosphatase, and LDH are unaffected (Bier *et al.*, 1971), indicating again the unique structure of complement molecules which governs oxidant sensitivity.

The values of oxidative destruction of complement remains uncertain. The apparent lack of toxicity in dogs in spite of suppression of both coagulation and complement (Bier *et al.*, 1971) suggests a role for NaOCl in modification of complement mediated disease or in-

flammatory states, yet the lack of specificity is all too apparent. Further work in experimental animals is needed.

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