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THE EFFECT OF IMMUNOSUPPRESSION ON THE DEVELOPMENT OF IMMUNITY TO FOWL TYPHOID

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ABSTRACT

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Chickens which were given cyclophosphamide during the first three to five days of life showed a marked depression in their humoral antibody response to sheep erythrocytes and *Brucella* antigen. Their ability to develop tuberculin sensitivity and their immune response to live *Salmonella gallinarum* vaccine was, however, unimpaired.

The administration of methylprednisolone acetate, hydrocortisone acetate, azathioprim and 6-mercaptopurine to either six or 10 to 12-week-old chickens neither selectively depressed the humoral or cellular immune response nor affected the immune response to live *S. gallinarum* vaccine. Similarly, the immune response could not be depressed by thymectomy or antilymphocyte globulin.

These results indicate that immunity to *S. gallinarum* after administration of a live avirulent vaccine is not dependent on a humoral immune mechanism but probably primarily on cellular immunity, although this could not be proven.

INTRODUCTION

A good immunity to fowl typhoid can be obtained by administering a live vaccine prepared from an avirulent rough mutant of *Salmonella gallinarum*. Fowls which have been immunized with such a vaccine exhibit a solid immunity to infection in the absence of any appreciable antibodies to 'O' antigen (Cameron, Fuls & Van Reenen, 1972). To explain this situation it was postulated that immunity was mediated either through serum antibodies, which are not detected by conventional serological tests, or through a true cellular mechanism (Cameron *et al.*, 1972).

To investigate this hypothesis it was decided to depress selectively either the humoral or cellular immune responses and study the resultant effect on the development of immunity to fowl typhoid.

Two distinct immunological systems have been identified in the chicken; cellular immune responses, as typified by graft rejection, are dependent on an intact thymus during ontogeny, while maturation of the antibody producing system is regulated by the bursa of Fabricius. The existence of these two systems can be demonstrated by neonatal surgical thymectomy or bursectomy (Warner, Szenberg & Burnet, 1962; Warner & Szenberg, 1962; Cooper, Peterson, South & Good, 1966; Glick, 1969). The development of the bursa can also be depressed by injecting testosterone *in ovo* (Meyer, Appaswamy Rao & Aspinall, 1959; Mueller, Wolfe & Meyer, 1960; Glick & Sadler, 1961; Warner, Uhr, Thorbecke & Ovary, 1969; Warner, Ovary & Kantor, 1971).

The immune response can also be modified by administering cytotoxic or immunosuppressive drugs and antilymphocyte globulin.

The nature and mode of action of the major immunosuppressive drugs have been reviewed by Gabrielsen & Good (1967). The immunosuppressants which have found the widest application are cyclophosphamide, azathioprim, 6-mercaptopurine and the cortico-steroid hormones. Cyclophosphamide has been found to alter the susceptibility of mice to viraemia (Du Buy, Worthington & Johnson, 1971) and also to depress the immune response to *Listeria monocytogenes* for longer than any other drug (Tripathy & Mackaness, 1969). Lemmel, Hurd & Ziff (1971) showed that cyclophosphamide restricted the development of auto-immune phenomena in mice while Linna, Frommel & Good (1972) obtained a spectacular depression of the humoral immune response of newly hatched chicks after administration of cyclophosphamide. Luckins (1969) and Dukor & Dietrich (1970) also obtained immunosuppression in mice to *Trypanosoma congolense* and heterologous erythrocytes by administering cyclophosphamide.

As already mentioned, azathioprim, 6-mercaptopurine and corticosteroids are well recognised immunosuppressants (Sato & Glick, 1970; Wilson & Frenkel, 1971; Berenbaum, 1971; North, 1971) and they were included in this study for comparative purposes.

Another method of immunosuppression is by the administration of antilymphocyte or antithymocyte serum or globulin (ALS/G or ATS/G). ALS is known to affect particularly the cellular immune responses such as graft rejection (Levey, 1970). It also provokes viral oncogenesis and potentiates *Mycobacterium* infections (Gauges & Rees, 1968; Hirsh, 1970) as well as listeriosis (Mackaness & Hill, 1969), and suppresses immunity to toxoplasmosis in hamsters (Centurier, 1970). Furthermore, rabbit antichicken thymocyte globulin in association with guinea pig complement, is also effective in preventing the development of allergic encephalomyelitis in chickens (Janković, Isaković, Petrović, Vujić & Horvat, 1970).

The most effective immunological depression in the chicken using ALS has, however, been obtained by a combination of thymectomy and duck anti-chicken ALS. This procedure not only inhibited graft rejection but also depressed antibody production against horse erythrocytes, although the development of anti-*Brucella* titres was unaffected (Rous & Warner, 1972).

MATERIALS AND METHODS

Experimental animals

Two categories of New Hampshire chickens were used: 10 to 12-week-old birds of from 1,0 to 1,3 kg mass and newly hatched chicks.

Thymectomy and bursectomy

Surgical thymectomy on day-old chicks was performed as described by Peterson, Burmester, Fredrickson, Purchase & Good (1964). A solution containing 7,5 mg thiopentone sodium and 2,5 mg pentobarbital sodium per 4 ml was used as anaesthetic: 75 mg Intraval sodium powder(*) was dissolved in 3,6 ml distilled water and mixed with 0,4 ml Sagatal(*). This solution was further diluted 1: 10 with distilled water and 0,2 to 0,5 ml injected intra-

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(*) Maybaker (SA) (Pty) Ltd, Port Elizabeth

muscularly. Hormonal bursectomy was accomplished by injecting 0,075 ml testosterone proprionate (50 mg/ml) (**) into the allantoic cavity of fertile eggs on the 12th day of incubation. This dosage (3,75 mg/egg) is in accordance with those used by Warner *et al.* (1969) and Warner *et al.* (1971). Unfortunately, as has also been found by Mueller *et al* (1970) and Warner *et al* (1962), the mortality rate in the hatched chicks was high and an insufficient number of healthy birds survived which could be used experimentally.

Immunosuppressive drugs

The following drugs were used in this study: azathioprim, 50 mg tablets [Imuran(*1)] cyclophosphamide [Endoxan(*2)]; 6-mercaptopurine, 50 mg tablets [Puri-Nethol(*3)]; methylprednisolone acetate, 20 or 40 mg/ml [Depo-Medrol(*4)] and hydrocortisone acetate 25 mg/ml [Cortril(*5)].

The drugs were prepared according to the manufacturer's recommendations and administered at the dosage levels by the routes and according to the schedules indicated in Tables 1, 2 and 3. The dosages were adjusted weekly in accordance with the average mass gain of each experimental group of chickens.

Antithymocyte globulins

Antiglobulin to chicken thymocytes was prepared in either goats, rabbits or ducks. Chicken thymocytes were obtained from 10-day-old chickens as described by Levey & Medawar (1966), except that the cells were suspended in Eagle's medium.

For preparation of goat antithymocyte globulin (GATG) thymocyte suspensions containing 10^9 cells/ml were injected into each of four goats according to the following schedule:

Day 1: 2,0 ml subcutaneously.
Day 10: 1,0 ml intravenously.
Day 20: 1,0 ml intravenously.
Day 28: 1,0 ml intravenously.
Day 35: Bleed.

The serum was inactivated at 56 °C for 30 min and consecutively absorbed with an equal volume of packed chicken erythrocytes at room temperature for 30 min and with fresh cells at 4 °C for 18 h.

Rabbit antithymocyte globulin (RATG) and duck antithymocyte globulin (DATG) were prepared according to the procedure of Levey & Medawar (1966).

Goat and rabbit globulins were purified by ammonium sulphate precipitation (Campbell, Garvey, Cremer & Sussdorf, 1964) while duck globulin was purified by sodium sulphate precipitation (Williams & Chase, 1967). In both instances the final precipitate was dissolved in phosphate buffered saline (pH 7,2) equal in volume to half the original volume of serum.

The agglutination and cytotoxicity titres of the final products were assayed as described by Abaza & Woodruff (1966). The agglutination titres were between 1: 192 and 1: 384 and the cytotoxicity titres between 1: 256 and 1: 512.

Antigens and tests

Experimental birds were immunized with *Mycobacterium avium*, *Brucella abortus* antigen, sheep erythrocytes (SRBC) and live *S. gallinarum* vaccine according to the schedules outlined below.

M. avium antigen was prepared by suspending heat killed bacilli (10 mg/ml) in incomplete Freund's adjuvant*. Each bird was given a single injection of 0,5 ml intraperitoneally (Warner *et al.*, 1962). Sensitivity was tested by injecting 0,05 ml avian tuberculin into a wattle. In older birds the increase in wattle thickness was measured 48 h later while in young chicks the extent of the reactions was expressed as 4+, 3+, 2+, 1+ and 0 by an unbiased colleague. The figures given in Tables 2 and 3 are the mean of these estimates.

Standard *B. abortus* antigen was diluted 1: 500 in saline and 0,5 ml injected intravenously; the birds were bled from the wing vein and the anti-*Brucella* agglutination titres determined by the method of Alton & Jones (1967).

For immunization with erythrocytes, 0,5 ml suspension of washed SRBC (0,1%) was injected intravenously. Anti-SRBC agglutination titres were determined by adding 0,05 ml of a 1,0 per cent suspension of SRBC to doubling dilutions of serum in microtitre plates. The end point of the titration was taken as the highest dilution showing complete agglutination after 60 min at room temperature.

The fowls were immunized against *S. gallinarum* infection by the subcutaneous injection of 2,0 ml of a suspension of the rough strain BUV containing $2,5 \times 10^8$ live bacteria/ml (Cameron *et al.*, 1972). The anti 'O' titres to the rough strain were determined and the immunized fowls challenged *per os* with 5×10^8 or $2,5 \times 10^8$ virulent bacteria (*S. gallinarum* strain BV 1007) (Cameron *et al.*, 1972).

The final figures for all the serological tests were obtained by calculating the geometric means.

Experimental design

Two experimental procedures were followed. The following schedule applied to 10 to 12-week-old fowls: *M. avium* was injected two weeks before administration of *B. abortus*, SRBC and *S. gallinarum* vaccine. Treatment with the various immunosuppressants commenced four days before immunization and continued until two days before challenge (Table 1).

The birds were first bled five days after immunization and again one day before challenge. The tuberculin tests were conducted two days before challenge and read after 48 h, i.e. just prior to challenge.

In the case of newly hatched chicks the following schedule was followed:

M. avium antigen as well as the first *B. abortus* and SRBC injections were given three weeks before challenge (three weeks of age). They were bled six days later and the second dose of *B. abortus*, SRBC as well as *S. gallinarum* vaccine inoculated on the following day. All the birds were bled for the second time after a further six days. The tuberculin tests were done three weeks after sensitization and read on the day of challenge, i.e. 14 days after immunization with *S. gallinarum* vaccine.

The immunosuppressant drugs were given according to the schedule shown in Table 2 and continued until the day before challenge where applicable.

RESULTS

The effect of the various immunosuppressive drugs on the immune response of 10 to 12-week old fowls is shown in Table 1.

(**) Propan Pharmaceuticals (Pty) Ltd, Wadeville, Germiston
(*1) Burroughs Wellcome & Co., London
(*2) Noristan Laboratories (Pty) Ltd, Silverton, Pretoria
(*3) Burroughs Wellcome & Co., London
(*4) Upjohn (Pty) Ltd, Isando
(*5) Pfizer Corporation, Brussels

* Difco Laboratories, Detroit, Michigan

TABLE 1 The effect of immunosuppressive drugs and GATG on the immune response of 10 to 12-week-old New Hampshire fowls

Group	No.	Immunosuppressant	Dosage & Route	Frequency	Mean anti SRBC titre			Mean anti *Brucella* titre			Mean anti *Salmonella* R '0' titre			Tuberculin reaction: Mean increase mm	Survivors after challenge
					First bleeding	Second bleeding	Mean	First bleeding	Second bleeding	Mean	First bleeding	Second bleeding	Mean		
1	6	Cyclophosphamide	30 mg/kg i.v.	Day −3 and 48 hourly	10	10	10	403	57	151	16	28	21	0,13	5/6
2	6	Cyclophosphamide	50 mg/kg i.v.	Day −3 and 48 hourly	18	3	7	508	57	170	3	3	3	0,3	0/2*
3	6	6-mercaptopurine	45 mg/kg p.o.	Day −3 and daily	6	36	15	1 140	57	254	25	45	34	0,07	5/6
4	7	6-mercaptopurine	80 mg/kg p.o.	Day −3 and daily	6	12	8	1 901	177	579	7	9	8	0,94	4/7
5	7	Methylprednisolone acetate	2 mg/kg i.m.	Day −3 and +5	15	15	15	1 737	173	955	7	32	15	0,19	6/6*
6	8	Methylprednisolone acetate	2 mg/kg i.m.	Day −3; Day 0 and Day +4	4	16	8	100	3	19	3	11	6	2,3	5/7*
7	7	GATG	1,0 ml i.v. 0,5 ml i.v. 0,5 ml i.m.	Day −3 Day −1 Day +1 and 48 hourly	6	8	7	1 561	143	472	7	32	15	0,09	6/6*
8	6	Combination of Methylprednisolone acetate & GATG	Combination of groups 5 & 7	Combination of groups 5 & 7	—	—	—	—	—	—	—	—	—	—	†
9	14	Antigens only	—	—	63	58	59	1 403	155	725	144	84	109	2,07	10/14
10	14	None	—	—	5,4	4,3	4,8	0	0	0	3,7	7,0	4,9	0,0	0/14

Day 0 = Day of immunization
i.v. = Intravenously
i.m. = Intramuscularly

p.o. = oral administration
s.c. = subcutaneously

* Remaining birds died before challenge
† All the birds died before challenge

TABLE 2 The effect of immunosuppressive drugs on the immune responce of 6-week-old New Hampshire chickens

Group	No.	Immunosuppressant	Dosage & Route	Frequency	Mean anti SRBC titre			Mean anti *Brucella* titre			Mean anti *Salmonella* R '0' titre			Tuberculin reaction: index	Survivors after challenge
					First bleeding	Second bleeding	Mean	First bleeding	Second bleeding	Mean	First bleeding	Second bleeding	Mean		
1	12	Cyclophosphamide.....	2 mg (66 mg/kg) i.p.	Day +1; +2; +3; +4 & +5 only..	5,0	45,2	15,1	3,9	8,8	5,8	22,5	90,5	45,1	2,6	7/8*
2	8	Cyclophosphamide.....	66 mg/kg i.p.....	As above and 3 times/ week.....	0,4	9,2	1,9	0,0	1,8	1,3	1,6	2,6	2,1	1,4	3/5*
3	12	Cyclophosphamide.....	2 mg (66 mg/kg) i.p. 4 mg (132 mg/kg) i.p.	Day 0 & Day +1 Day +2 & Day +3 only	4,4	24,7	10,4	4,9	6,9	5,8	2,3	6,6	3,9	1,9	8/11*
4	10	Methylprednisolone acetate.....	0,4 mg s.c.....	Day 0 & weekly....	7,3	76,1	23,6	12,6	12,6	12,6	5,4	38,0	14,3	1,2	5/8*
5	10	Hydrocortizone acetate.....	0,5 mg s.c.....	Day 0 & +3 times/ /week	2,8	21,1	7,7	0	0	0	2,0	4,0	2,9	0,2	2/4**
6	10	Azathioprim.....	0,5 mg (±15 mg/kg) p.o.	Day 0 & daily.....	225,7	332,5	269,9	96,6	80,0	87,7	41,2	29,3	34,8	2,1	9/10
7	12	Azathioprim.....	1,5 mg (50 mg/kg) p.o.	Day 0 & daily.....	33,7	90,5	55,3	18,2	89,8	40,4	21,1	215,2	67,3	3,5	9/12
8	12	6-Mercaptopurine.....	3 mg (100 mg/kg) p.o.	Day 0 & daily.....	24,0	40,3	31,1	6,0	10,2	7,8	16,0	32,0	22,6	3,1	12/12
9	29	Antigens only.....	3 mg (100 mg/kg) p.o.	Day 0 & daily.....	71,9	283,3	129,5	144,6	106,7	115,1	59,8	121,9	63,3	2,0	21/29
10	30	None.....	3 mg (100 mg/kg) p.o.	Day 0 & daily.....	1,4	2,5	1,9	0	0	0	1,7	2,6	2,1	0,0	2/30

Day 0 = Day of hatching

p.o. = Oral administration
s.c. = subcutaneously
i.p. = Intraperitoneally

* Remaining chickens died before challenge
** Severely stunted

TABLE 3 The effect of ATG on the immune response of 6-week-old New Hampshire chickens

Group	No.	Immunosuppressant	Dosage & Route	Frequency	Mean anti SRBC titre			Mean anti *Brucella* titre			Mean anti *Salmonella* R '0' titre			Tuberculin reaction: index	Survivors after challenge
					First bleeding	Second bleeding	Mean	First bleeding	Second bleeding	Mean	First bleeding	Second bleeding	Mean		
1	10	RATG.....	0,5 ml s.c..... 2,0 ml s.c.....	Day +1 & +4.... Day +23 & +26..	7,4	11,3	9,2	117,5	67,3	88,9	4,8	40,3	13,9	2,3	6/9*
2	12	RATG.....	0,5 ml s.c..... 1,0 ml s.c..... 1,5 ml s.c.....	Day +2 & +6..... Day +17 & +20... Day +23 & +26...	50,8	120,8	78,3	75,5	181,4	117,0	32,0	199,0	79,8	3,1	10/12
3	11	DATG.....	0,5 ml s.c..... 2,0 ml s.c.....	Day +1 & +4..... Day +23 & +26...	24,3	15,1	19,1	52,7	124,4	81,0	—	10,9	—	1,0	10/11
4	12	Thymectomy & DATG.....	Thymectomy..... 0,5 ml s.c..... 1,0 ml s.c..... 1,5 ml s.c.....	Day +1..... Day +2 & +6..... Day +17 & +20... Day +23 & +26...	71,2	111,4	89,0	34,1	54,4	43,1	56,4	93,8	72,7	3,5	10/12
5	20	Antigens only.....	—	—	66,5	256,0	132,0	97,4	114,5	97,2	40,3	133,5	128,0	2,1	17/20
6	20	None.....	—	—	1,2	2,4	1,7	0	0	0	1,5	3,4	2,2	0	6/20

* One chicken died before challenge

Day 0 = Day of hatching
s.c. = Subcutaneously

All the drugs caused some depression of the antibody response, especially to SRBC and the 'O' antigens of the rough *S. gallinarum* vaccine strains, while methylprednisolone acetate also depressed the response to *B. abortus* antigen.

The tuberculin reaction was also depressed in all the groups except the group which received methylprednisolone acetate in which the mean increase was comparable to that of the controls. It should, however, be pointed out that the reactions of individual birds varied greatly which casts some doubt on the validity of the results. This is borne out by the discrepancy between these values for the two groups given six mercaptopurine and the fact that the depression of the tuberculin reaction with GATG could not be repeated in later experiments using rabbit ATG or duck ATG.

The fact that none of the treatments gave a clear selective depression of either the humoral (e.g. anti *Brucella* titres) or cellular (e.g. tuberculin reaction) is nevertheless worthy of note. Furthermore, there was no impairment of the development of immunity to fowl typhoid following the administration of live vaccine.

Since experiments using higher doses of drugs in 10 to 12-week-old fowls become impractical, subsequent investigations were done in young chickens. The effects of different immunosuppressive drugs administered from the day of hatching are compared in Table 2.

The most notable result was the consistent and pronounced selective depression of the humoral immune response following treatment with cyclophosphamide. These findings substantiate those obtained by Linna *et al* (1972).

With the exception of hydrocortisone acetate where the dose was beyond the limits of tolerance, none of the other drugs materially influenced the tuberculin reaction.

It should be noted that although cyclophosphamide caused such a marked inhibition of the antibody response, the development of immunity to fowl typhoid was unaffected.

The results of attempts to depress the cellular immune response in 6-week-old chickens specifically are shown in Table 3.

Despite the use of various procedures which have reputedly depressed the cellular immune reactions, the tuberculin reaction was unaffected. Similarly no deleterious effect on the immunity to fowl typhoid was observed in any of the groups.

Discussion

The results presented in this paper indicate that the immunity to fowl typhoid which is evoked by a live *S. gallinarum* vaccine are not dependent on circulating serum antibodies. This conclusion can be drawn from the fact that a very good immunity can be established in chickens whose humoral immune response has been severely depressed by cyclophosphamide given during the first week of life.

The reverse, i.e. that immunity to fowl typhoid is decreased concomitantly with depression of the cellular immune mechanisms, could not be proven. However, not all cellular immune states are necessarily depressed by the procedures employed. Although graft rejection is for instance depressed after thymectomy, the tuberculin reaction which is also a cellular immune response remains intact (Cheville & Richards, 1971; Panigrahi, Fauser, Mallmann & Waxler, 1972). Moreover, North (1973) has shown that thymectomy in mice suppressed the response to tuberculosis while the immune response to *Listeria* infection was unaffected. Both these phenomena are cellular immune reactions and they concluded that there is a quantitative difference between various manifestations of cellular immunity.

Although the immunity to fowl typhoid is not impaired by thymectomy combined with ALG administration, it does not necessarily mean that a cellular immunity is not involved. Experiments on the passive transfer of immunity to fowl typhoid by serum or cells should elucidate this question.

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