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Subclasses of IgG in different aged rats with fasciolosis

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Abstract. The variations in antibody responses (total IgG and IgG1, IgG2a, IgG2b, and IgG2c subclasses) were studied in two groups of rats infected with metacercariae of the trematode *Fasciola hepatica* L. Animals of group 1 were 4 weeks old, and rats of group 2 were 13 weeks old. All IgG subclasses increased during the course of infection except IgG2c, which decreased. The younger rats reached more marked responses than the older, at least during the period of this trial. IgG1 and IgG2a antibodies reached the highest levels, and among these two, IgG2a response was slightly superior to IgG1.

During the infection with the trematode *Fasciola hepatica* L., rat develops a strong immunological response against some excretory-secretory products (Hughes et al. 1981). Poitou et al. (1993) reported that sequential release of *F. hepatica* antigens, associated with the developmental stages of the parasite, induced successive humoral responses and lymphocyte stimulations, which resulted in eosinophilia, neutrophilia and elevated serum IgE and IgG (IgG1 and IgG2a) levels. The adherence of eosinophils and neutrophils to flukes *in vitro* has been demonstrated by Doy et al. (1980), and the attachment of rat granulocytes to flukes has been shown to be antibody dependent.

In the present study we have investigated the different IgG subclasses (IgG1, 2a, 2b and 2c), involved in antibody responses in two groups of rats with different age infected with *F. hepatica*. The objectives were (a) to investigate if all IgG subclasses showed similar kinetics; (b) to evaluate if these changes could be age-related; and (c) to gain more information about the immune response which develops during an experimental fasciolosis.

MATERIALS AND METHODS

Sprague-Dawley female rats were used in this trial as hosts for *Fasciola hepatica* infection; these animals were maintained in a room electrically heated (22 ± 1°C) with a 12 h light-dark cycle. Food and water were available *ad libitum*. Metacercarie of the trematode were obtained after infection of *Lymnaea truncatula* L. snails with *F. hepatica* miracidia, obtained from eggs collected from livers of slaughtered sheep. The viability of metacercariae was estimated *in vitro*, prior to infection. Each rat received, by gastric tubing, 20 metacercariae. Rats were divided into three groups of 9 rats in each: Group 1 (G-1) infected when the animals were 4 weeks old (80-120 g), and Group 2 (G-2) when 13 weeks old (200-240 g). Another group (4 and 13 weeks old rats) was left as an uninfected control (G-C).

All rats were bled weekly, by retro-orbital venous sinus puncture under anaesthesia, from one week prior to infection to the 21st week after infection (a.i.), to evaluate the immunological response. Systemic antibody responses were established by detecting IgG against the excretory-secretory (ES) antigen from *F. hepatica* adults using an indirect-ELISA. This antigen was obtained from adult flukes collected from cattle bile ducts and washing them several times in phosphate-buffered saline (PBS), and maintaining in this medium at 37°C for 3 hours. Eggs were removed by sieving and the ES products collected. The medium was centrifuged 50 min at 4°C and 17,000 g, and the supernatant was collected, concentrated and stored lyophilised. The protein concentration was estimated by the BCA technique described by Pierce®, using a concentration of 3.5 $\mu g \cdot ml^{-1}$ to coat the wells of ELISA plates, according to Sánchez-Andrade (1994).

Wells of micro-ELISA plates were coated with 100 µl ES (3.5 µg protein ml^{-1}) and incubated for 3 hours at 37°C. After two washes, blocking of excess-binding sites was performed by incubation with 300 µl of PBS containing 0.05% Tween and 1% skimmed milk (PTM) for 2 hours at 37°C. After two washes, rat sera diluted (1/20) in PTM were added in duplicate to the wells and incubated for 1 h at 37°C. After 6 washes, 100 µl of horseradish-peroxidase-conjugated (HRP-conjugated) rabbit anti-rat IgG, IgG1, IgG2a, IgG2b and IgG2c (H&L, Nordic Immunological Laboratories) were added at a dilution of 1/1,500, 1/3,000, 1/2,000, 1/2,000, 1/2,000, respectively, and incubated for 1h at 37°C. After 6 washes, 100 µl of substrate consisting of 10 mg of ortho-phenylenediamine, 12 ml citrate buffer and 10 µl of 30% hydrogen peroxide were added to each well. The plate was incubated in the dark for 10 min at room temperature. The colour reaction was stopped by addition of 100 µl of 3N sulphuric acid, and absorbances were read using a spectrophotometer (Titertek Multiskan) at 450 nm. Serum pools from 4 uninfected rats and 4 infected ones were used as negative and positive controls, respectively.

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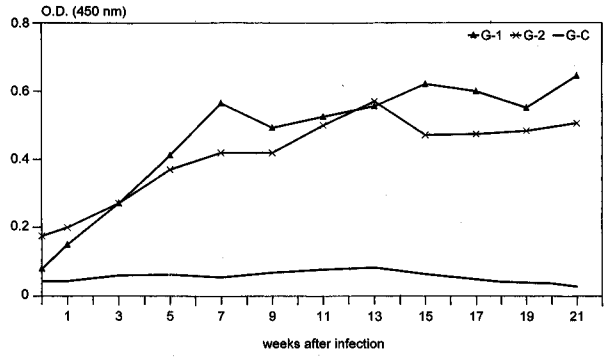

Fig. 1. Anti-ES IgG levels in sera from rats infected with metacercariae of *Fasciola hepatica*. G-1 – rats infected at 4 weeks old; G-2 – rats infected at 13 weeks old; G-C – control group.

RESULTS

The total IgG response was detected as soon as week 2 after infection (a.i.) in both infected groups (Fig. 1) and absorbance values were significantly higher than in the controls ($p < 0.05$). The optical density (OD) values were always slightly superior in G-1 from the 1st week a.i., but they rose progressively in G-1 and G-2 and remained elevated until the end of the study. No age-related effect was observed between G-1 and G-2, and no statistically differences were appreciated ($p > 0.05$).

The IgG1 subclass began to increase at week 5 a.i. in both infected groups (Fig. 2a), peaked on 19 weeks a.i., remaining high from week 9 a.i., and did not decrease towards the end of the study. The IgG2a and IgG2b isotypes showed an earlier and more marked increase than IgG1 (Fig. 2b,c), also presented elevated levels from week 3-5 a.i. and reached one peak at 10-11 week a.i. It was noticed that the level of both IgG decreased from the 15th week a.i. However, the IgG2c subclass response was different to the IgG1, IgG2a and IgG2b (Fig. 2d) since a plateau was achieved both in G-1 and G-2 from the first week a.i. to the 11-13, when it began to decrease until the end of the study. This IgG2c isotype was the unique that did not increase during the course of the infection.

In respect to the intensity of the IgG subclass responses, we can clearly appreciate that IgG1 and IgG2a reached the highest levels, and among these two, it seems that IgG2a response was slightly superior to IgG1. By means of the Mann-Whitney test we proved that difference age-related kinetics of IgG isotypes were statistically significant in IgG1, IgG2a and IgG2c, among the two groups of infected rats. Likewise, we proved that IgG2a subclass exhibited significant differences only during the acute phase of infection.

By means of the Spearmann's rank correlation test we proved a significant correlation ($p < 0.05$) between G-1 and G-2, in IgG1 and IgG2a curves.

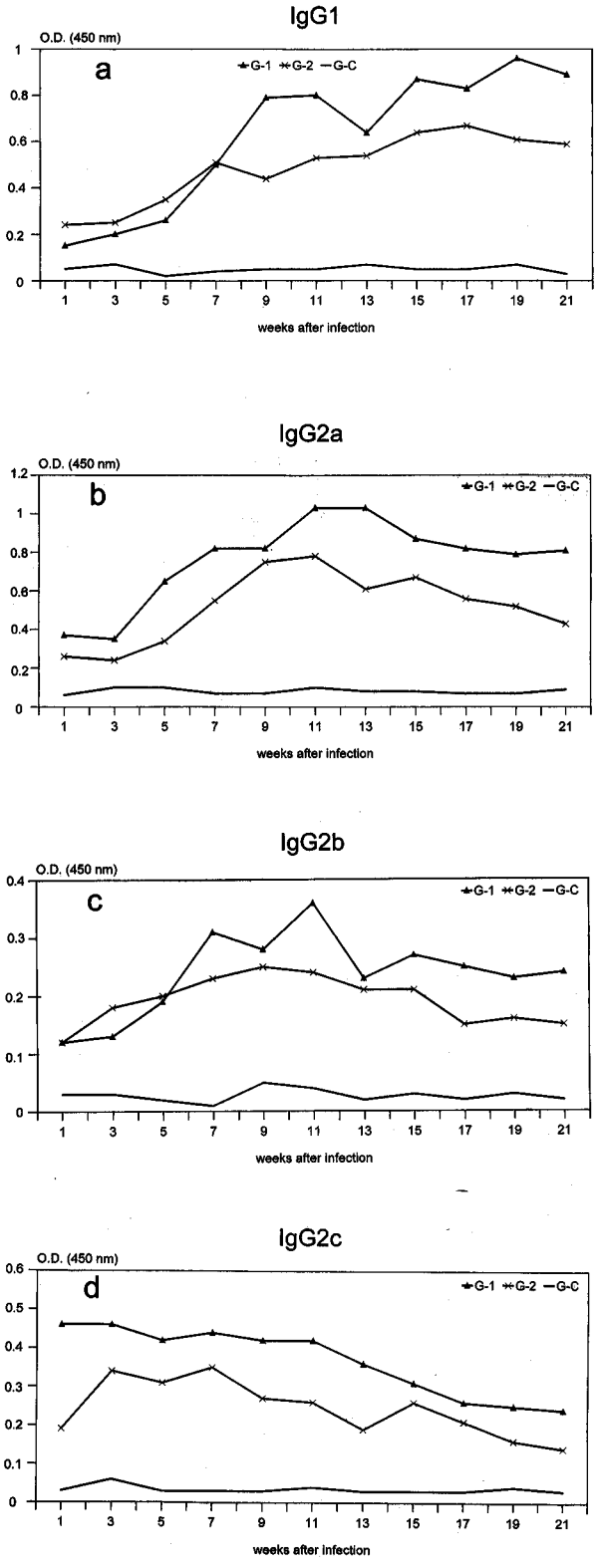

Fig 2. IgG subclass responses to ES-*Fasciola hepatica* antigen over the course of infection, in rat serum. G-1 – rats infected at 4 weeks old; G-2 – rats infected at 13 weeks old; G-C – control group. a) IgG1 response; b) IgG2a response; c) IgG2b response; d) IgG2c response.

DISCUSSION

Pfister et al. (1984), Poitou et al. (1992, 1993) and Paz et al. (1997) pointed out that *Fasciola hepatica* in rat induces early appearance of total IgG responses against excretory-secretory antigens. Nevertheless, little information has been reported about the IgG subclasses involved in rat fasciolosis. Grzych et al. (1984), Horta and Ramalho-Pinto (1984) and Khalife et al. (1985a, b) observed that IgG1, IgG2a, IgG2b and IgG2c could be evaluated in rats infected with *Schistosoma mansoni*, and Poitou et al. (1993) studied the kinetics of IgG1 and IgG2a in rats experimentally infected with *F. hepatica*. We demonstrated that IgG1, IgG2a, IgG2b and IgG2c were present during infection with *F. hepatica* in rats. The response of IgG2a and IgG2b increased earlier and more quickly than IgG1, which partially coincided with Poitou et al. (1993). IgG2a was the most abundant immunoglobulin, as occurs in rat schistosomosis (Horta and Ramalho-Pinto 1984, Capron et al. 1987). IgG2c, on the contrary to the other IgG subclasses, did not increase from the 1st week a.i., and decreased from week 11-13 a.i. to the end of the trial, in coincidence with that reported by Khalife et al. (1985b).

Horta and Ramalho-Pinto (1984) pointed that IgG2a and/or IgG2b were involved in protective immunity to *Schistosoma mansoni* in rats, possibly through a complement-mediated mechanism. Indeed, Khalife et al. (1985a, b) and Capron et al. (1987) demonstrated that IgG2a was detectable on the surface of eosinophils at the early stage of some parasite infections. These authors observed that IgG2a was involved in antibody-dependent mediated cytotoxicity (ADCC), because this immunoglobulin presented anaphylactic activity through the release of several granule components (eosinophil peroxidase, cationic proteins). Nevertheless, IgG2c did not exhibit any killing activity *in vitro* against schistosomula, and Grzych et al. (1984) and Khalife et al. (1985a,b) showed that IgG2c played a blocking activity both *in vitro* and *in vivo* in *S. mansoni*, inhibiting specifically the IgG2a-mediated eosinophil peroxidase (EPO) release. Hanna (1980), Doy et al. (1981) and Glauert et al. (1985) noticed that eosinophils and neutrophils attached to flukes, and this phenomenon was antibody-dependent. Although ADCC appears to be the main parasite-killing mechanism in rat and human schistosomosis (Capron et al. 1987), we observed egg-output by coprological analysis (data not shown), so the immune response that takes place seems unable to prevent the development of fasciolosis. Glauert et al. (1985) reported that eosinophils were unable to kill juvenile *F. hepatica* in the presence of specific antiserum, due to the presence of a protective layer, consisting of antigen/antibody complexes, on the parasite's surface.

Our results concerning on age-related changes during the first 21 weeks a.i. in infected rat pointed out that young rats exhibited more marked responses of IgG subclasses. These results agree with those obtained by Dobber et al. (1995), who related them with the differentiation stage of B cells.

We can conclude that primary experimental infection of rats with *F. hepatica* stimulates a marked immune response including the production of the rat immunoglobulin G1, IgG2a, IgG2b and IgG2c. Nevertheless, *F. hepatica* is able to evade this immune host defence. Several differences were found when age was considered, showing that young rats exhibited the most marked responses in the study of IgG subclass curves, and these differences were significant ($p < 0.05$).

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