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ANTIBODY-MEDIATED RESPONSE OF PIGEONS TO ARGAS POLONICUS LARVAL FEEDING AND CHARACTERIZATION OF LARVAL ANTIGEN

F. DUSBÁBEK, Š. LUKEŠ, V. MAŤHA[1] and L. GRUBHOFFER

Institute of Parasitology and [1]Institute of Entomology, Czechoslovak Academy of Sciences, České Budějovice, Czechoslovakia

Abstract. Circulating antibodies to larval *Argas polonicus* antigen detected in the blood of pigeons by means of ELISA reach their highest level 3–6 days post-tick attachment. During 6–8 days post infestation when most larvae detach from their host, there is an abrupt drop of the antibody level in blood followed by second peak at day 10–15. During the secondary and subsequent infestations the dynamics of the antibody production is analogous, but the maximum absorbance values found are higher with each following infestation. This is in direct correlation with the growth of immune resistance of hosts. The transfer of immunoglobulins of resistant pigeons produces in naive hosts a partial resistance manifested in a statistically significant ($P < 0.01$) reduction of the number of engorged larvae, in the shortening of larval feeding period and in the decrease of their mean weight after feeding. However this resistance was significantly ($P < 0.01$) less expressed than in naturally resistant pigeons during secondary infestation. The protracted effect on the duration of premoulting period and the percentage of moulted larvae manifested in larvae after secondary infestation was not apparent in larvae fed on immunoglobulin recipients. Six major protein components from larval extract having molecular weight of approximately 19, 21, 23, 27, 45 and 165 kilodaltons, were recognized by serum of resitant pigeons.

Acquired resistance of laboratory animals to tick infestation has an immunological basis consisting of cell-mediated, antibody-mediated and complement-dependent effector mechanisms (Wikel and Whelen 1986). The antibody-mediated response of hosts to tick feeding has been proved by detection of circulating anti-tick antibodies in infested hosts and by successful passive transfer of resistance by serum from resistant to naive hosts (Wikel and Allen 1982, Allen 1987). In some cases, however, only partial or no resistance has been obtained by recipients of immune plasma or serum. The failure in these experiments indicates that both the immune serum factors and immune cells may be necessary to achieve complete resistance (Askenase et al. 1982, Wikel and Allen 1982).

The circulating antibodies were detected in rabbits and guinea pigs after *Ornithodoros moubata* feeding (Brossard et al. 1981, Centurier et al. 1981), but no attempts were made at passive transfer of immune resistance to argasids from host to host.

Since the pioneer work of Trager (1940) no acquired resistance of bird hosts to argasid ticks had been reliably confirmed until the results of investigations on acquired resistance of pigeons to *Argas polonicus* were published by Dusbábek and Škárková-Špaková (1988). In this study also the circulating anti-tick antibodies in serum of resistant pigeons were detected by means of counter current immunoelectrophoresis, using the extract from whole larvae and salivary glands of adults as antigen. Cutaneous response of sensitized pigeons to *A. polonicus* larval feeding was studied by Dusbábek et al. (1988) and characterized as cutaneous basophil hypersensitivity reaction.

Several proteins were recognized by serum of resistant hosts as antigens in salivary gland-, organ- or whole larvae-derived materials of hard ticks (Whelen et al. 1984, Wikel and Whelen 1986, Brown and Askenase 1986, Shapiro et al. 1986, 1987, Gordon and Allen 1987), having the molecular weights of 16–172 kilodaltons.

Unfortunately no attempts were made to characterize the antigens produced by argasid ticks.

In the present paper we try to characterize the larval antigens of *A. polonicus* and define the role of antibody-mediated host immune response in the mechanism of acquired resistance of pigeons to this argasid.

MATERIALS AND METHODS

Larvae of the fourth laboratory generation of *Argas* (*Argas*) *polonicus* Siuda, Hoogstraal, Clifford et Wassef, 1979 from a colony of the Institute of Parasitology, Czechoslovak Academy of Sciences, České Budějovice, obtained originally from the type locality at Krakow, Poland, were used in our experiments. The ticks were maintained in cotton plug-topped vials inside glass jars over a saturated solution of NaCl at 27 ± 1 °C and 75 ± 5 % RH in darkness. One hundred of 30—60 day-old larvae were used for each experimental infestation.

Seventeen urban feral pigeons captured beyond the distribution area of *A. polonicus* were used as hosts. Seven pigeons were treated with 1 ml of immunoglobulins (total protein content 8.63 mg . ml^{-1}) isolated from the serum of pigeons sensitized by five subsequent infestations of argasid larvae at two-week intervals. The immunoglobulins were administered partly to brachial vena, partly to pectoral muscle of each pigeon. Five of them were infested with argasid larvae, two served as treated control. The other three pigeons served as untreated control in this experiment. The remaining seven pigeons were used for study of dynamics of circulating anti-tick antibodies in peripheral blood during natural infestations. The number of successfully engorged larvae, total weight of engorged larvae detached from hosts on individual days, duration of feeding and premoulting periods and percentage of moulted larvae were recorded.

For detection of anti-tick circulating antibodies in serum the common method of ELISA according to Voller et al. (1979) was used. Serum samples were diluted 1 : 50 in phosphate buffered saline pH 7.4 with 0.05 % Tween-20 and incubated for 1 hour at 37 °C on plates (M 29, Dynatech), coated with antigen diluted on 2.5 μg . ml^{-1} final protein content with coating buffer (sodium bicarbonate pH 9.6). After washing 100 μl of 1 : 40 diluted conjugate (rabbit anti-pigeon immunoglobulin peroxidase conjugate) was added and incubated for 1 hour at 37 °C. O-phenylenediamine (Lachema, Brno) in phosphate-citrate buffer (pH 5.0) served as substrate. Reaction was stopped with 2.5 M sulphuric acid after 15 min. incubation in darkness under laboratory temperature. Results were read on MR 580 microplate reader on line connected with Apple II computer. Intensity of reaction was recorded as absorbance at 492 nm. Each serum sample was tested twice.

Pigeon immunoglobulins both, from the serum of sensitized pigeons for experiments on passive transfer of immune resistance, or from the serum of naive pigeons for rabbit immunization, were prepared as an ammonium sulphate precipitate of whole pigeon serum and desalined by the rapid column chromatography method on Sephadex G-25 (Hudson and Hay 1980). Rabbit immunization against pigeon immunoglobulins was performed by double subcutaneous administration of 250 μg of naive pigeon immunoglobulins together with 1 ml of Al-Span-Oil adjuvants (SEVAC, Prague) at two-week interval. The rabbit anti-pigeon immunoglobulins were then separated from the rabbit serum by the same method and the conjugate was prepared using double-step glutaraldehyde methods after Avrameas and Ternynck (1971).

The whole larval extract in 0.15 M phosphate buffered saline (pH 7.4) was used as antigen in final protein content 2.5 μg . ml^{-1} for ELISA test. Antigens for polyacrylamide gel electrophoresis were prepared from 50 *A. polonicus* larvae homogenized in 500 μl of SDS reducing sample buffer (Laemmli 1970), then centrifuged for 10 min. at 5.000 g at 4 °C. Four two-fold diluted concentrations of homogenate supernatant were used, starting from concentration corresponding approximately to five larvae per gel house.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was used for antigen proteins separation. SDS-PAGE was performed in 10 % homogenous running gel after Laemmli (1970). An amount of 30 μl of larval homogenate was mixed 1 : 1 with sample buffer and heated at 100 °C for 3 min. The samples were double applied on the surface of the gel and electrophorized on Pharmacia GE 2/4 vertical slab electrophoresis apparatus under constant voltage 120 V until the tracking dye reached the bottom of gel. Proteins were detected by staining with Coomassie brilliant blue R-250. Pharmacia low molecular calibration kit was used to determine the molecular weights of separated proteins.

For antigen detection the blotting method of Towbin et al. (1979) was used. After the electrophoresis the gel was immersed in blotting buffer (0.025 M Tris, 0.192 M glycine, 20 % methanol, and 0.04 % SDS) for 30 min., then the separate proteins were electroblotted to nitrocellulose membrane. After this procedure the nitrocellulose sheet was immediately immersed in 5 % skim milk in phosphate buffered saline (PBS) containing 0.05 % Tween-20 (30 min.), then five times washed with

PBS-Tween and incubated with horseradish peroxidase-conjugated rabbit anti-pigeon immunoglobulins at a dilution of 1 : 20 for 45 min. Thereafter, the blots were washed as before and antigen-antibody complex visualized by incubating in substrate solution (12.5 mg of 3.3-diaminobenzidine, 15 μl of 30 % H_2O_2 in 50 ml of 0.1 M Tris-HCl buffer pH 7.6). The procedure was stopped by 0.5 % NaN_3 after a good contrast of zones was reached.

χ^2 (chi-square) calculation and Student's t-test were used for the statistical analysis of the results.

RESULTS

A. Dynamics of anti-tick circulating antibody production

Increased absorbance was detected in the sera of several naive hosts by means of ELISA as early as on day 2 post infestation with larval ticks and reached its first peak in individual experimental pigeons between days 3 and 6. There was a marked decrease of circulating antibodies between days 6 and 8 followed by moderate increase of their levels reaching the second peak at day 10—15 (Fig. 1). At secondary

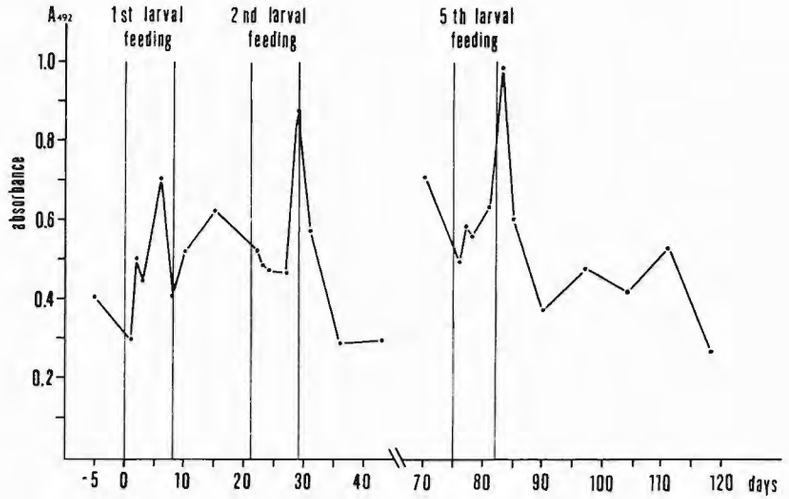

Fig. 1. Dynamics of circulating antibody formation in pigeons repeatedly infested with *Argas* (*A.*) *polonicus* larvae (ELISA, mean absorbance values at 492 nm).

and subsequent infestations the dynamics of antibody production had a similar tendency; however, the antibody level increased in individual experimental pigeons within 4—6 days post infestation, with a first maximum of antibodies between days 6 and 8 and an abrupt drop at about day 10. The values of maximum absorbance at second and fifth infestation were higher than those at primary infestation (Fig. 1).

After intravenous/intramuscular administration of immunoglobulins of resistant pigeons the recipients showed an increase of antibody levels on day 2 after application, with the maximum about day 3 and an abrupt drop as early as day 6 after application (Fig. 2A). Maximum values of antibody absorbance were considerably higher than in pigeons primary infested with larval ticks. In pigeons, to which immunoglobulins were administered at the simultaneous larval infestation, the level of maximum absorbance was also higher than in primary infested naive pigeons. In these

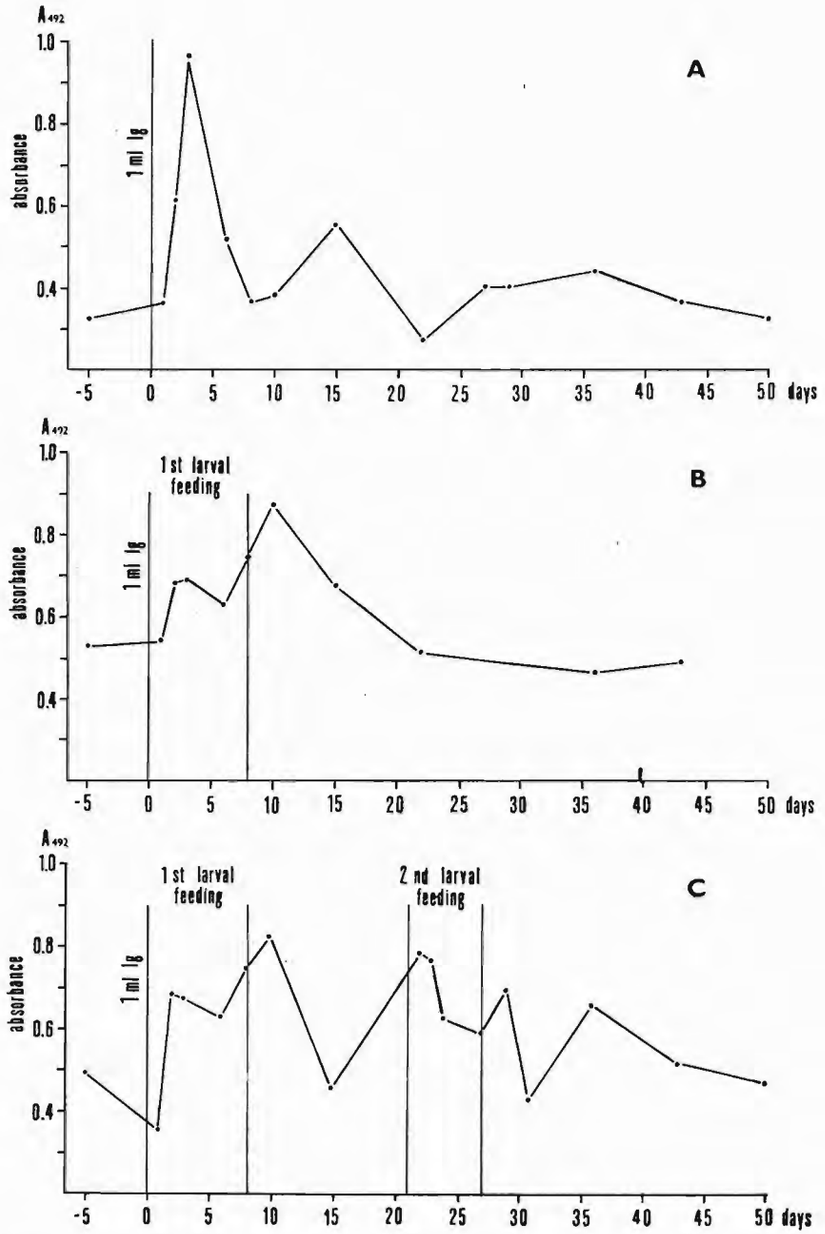

Fig. 2. Dynamics of circulating antibodies in pigeon recipients of immunoglobulins from the sera of resistant hosts. A — pigeon recipients of immunoglobulins without tick infestation; B — pigeon recipients of immunoglobulins infested simultaneously with *Argas (A.) polonicus* larvae; C — pigeon recipients of immunoglobulins repeatedly infested with *A. (A.) polonicus* larvae (ELISA, mean values of absorbance at 492 nm).

cases two absorbance peaks were recorded, the first within 2—3 days, the second about day 10 post infestation with consecutive antibody decrease about day 15. During the secondary infestation of treated pigeons the values of antibody absorbance did not reach the level detected at the primary infestation and were approximately at the level of secondary infestation of naive pigeons (Fig. 2B, C).

B. Passive transfer of immune resistance

By means of intravenous/intramuscular administration of immunoglobulins from resistant pigeons to naive hosts a partial immune resistance could be induced, manifesting itself in the reduced number of fed larvae, in the decrease of their weight and in the shorter feeding period on host (Table 1). These parameters were markedly lower ($P < 0.01$) in treated pigeons than in control pigeons, but markedly higher ($P < 0.01$) than at the secondary infested control pigeons, which had been actively sensitized with larval ticks at the primary infestation. In three out of five experimental pigeons these differences in the number of rejected larvae, the weight of engorged larvae and the feeding period as revealed in treated and control pigeons were very prominent, but in two cases no detectable immune resitance was induced by transfer of immunoglobulins. In the next two parameters observed — the duration of premoulting period and the percentage of moulted larvae — the influence of passive transfer of immune resistance in recipients did not show despite the fact that after active immunization with larvae during primary infestation there was a significant ($P < 0.01$) decrease of their values in both, the control and experimental pigeons (Table 1).

Table 1. Comparison of feeding success and future development of larvae of *Argas polonicus* fed on control pigeons and on recipients of immunoglobulins from immune serum (meann ± SD)

	1st feeding		2nd feeding	
	Control	Ig-recipients	Control	Ig-recipients
Larvae engorged (%)	87.5	66.3	36.0	17.7
Mean weight (mg)	2.16 ± 0.06	2.10 ± 0.15	1.85 ± 0.16	2.02 ± 0.18
Feeding period (days)	6.36 ± 0.64	6.26 ± 0.63	6.14 ± 0.70	6.15 ± 0.57
Premoulting period (days)	13.12 ± 1.48	13.48 ± 2.46	9.42 ± 1.23	9.90 ± 1.94
Larvae moulted (%)	94.29	94.97	81.94	92.45

C. Immunoblotting studies

By method of immunoblotting 14 distinct antigenic fractions were demonstrated in the homogenate of *A. polonicus* larvae, their molecular weight being from 165 to 19 kilodaltons, while six of them with molecular weight about 165, 45, 27, 23, 21 and 19 kilodaltons represented antigenically dominant fractions (Fig. 3). The comparison of nitrocellulose replica to homogenate proteins stained with Coomassie brilliant blue under conditions of SDS-PAGE shows clearly that these dominant fractions agree with the corresponding protein fractions of the homogenate (Fig. 3).

DISCUSSION

Argas polonicus larvae feed on pigeon hosts for 4—9 days; about 70% engorge to repletion within 6 days and over 90 % within 7 days (Dusbábek and Škárková—

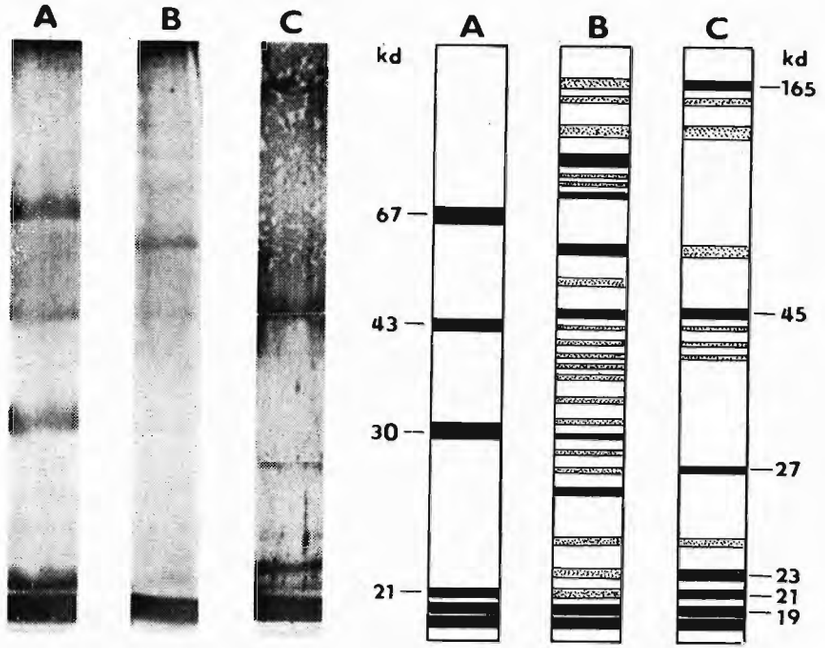

Fig. 3. Protein profile and immunoblot of fractionated *Argas* (*A.*) *polonicus* larval extract. A — Pharmacia low molecular calibration kit; B — SDS-PAGE stained with Coomassie brilliant blue; C — immunoblot analysis of proteins recognized by serum of resistant pigeon.

Špaková 1988). This rhythm of feeding agrees with the dynamics of the production of anti-tick circulating antibodies in naive pigeons. In our experiment the first, probably nonspecific antibodies could be detected by ELISA method since day 2 post-infestation and reached their highest levels in individual experimental pigeons between days 3 and 6. Between days 6 and 8, in some experimental pigeons as late as day 10, a marked drop of their levels was noted and followed by moderate increase of the level of probably specific antibodies until day 15. The kinetics of the antibody response at secondary and subsequent infestations ran a similar course, but the increase of antibody level was higher. At the secondary infestation the absorbance values were higher by 25 %, at the fifth infestation they were higher by almost 30 % than at primary infestation. This is in direct correlation with the growth of acquired resitance of hosts to larval ticks. Comparable results were obtained by Fujisaki (1977) and Fujisaki et al. (1980) in the studies on the production of precipitating and complement-fixing antibodies in rabbits infested with *Haemaphysalis longicornis*. These authors also recorded a gradual increase of antibody titres with the increasing number of infestations and a decrease of titres of precipitating antibodies in the period between infestations. The increase of titres of the two types of antibodies was detected as late as in the second week after primary tick infestation, namely in the period following their detachment, and the decrease of antibody titres was much slower — taking several weeks at the second and third infestations. Likewise Brossard and Girardin (1979) detected increasing titres of specific IgG in the sera of rabbits as late as at the end of the feeding of *Ixodes ricinus* females on their host. These differences may have

resulted from the methods used and from the entire arrangement of the experiment, but they may also have been due to the different species of tick and host as study objects. Brown et al. (1983) pointed out the differences in the blood basophil kinetics in hosts parasitized by ixodid and argasid tisks and McLaren et al. (1983) called attention to the differences in cutaneous cellular response in guinea pigs infested with ixodid and argasid ticks. Guttman et al. (1971) who studied antibody response of pigeons on bovine serum albumin (BSA) as a model antigen, found a relatively weak reaction by means of indirect haemagglutination and ring tests after the first administration of antigen and an accelerated onset of reaction as well as an increase of antibody titres after the second and third administrations of antigen. Similarly as in our experiments these authors detected increased antibody titres as early as from day 4 and a moderate decrease of titres on day 8 after administration of antigen. The influence of reactivity of different host as well as tick species from a different family is consequently the most probable.

Antigen presenting cells such as Langerhans cells in particular and probably macrophages as well, have been shown to bind tick antigen and are undoubtedly involved in the sensitization process in guinea pigs (Allen et al. 1979). Langerhans cells were shown to be capable of presenting tick salivary antigen to syngeneic lymphocytes (Nithiuthai and Allen 1985, Allen 1986). A similar system simulating adjuvant effect, gradually releasing tick antigens is apparently activated in pigeons as well and may be responsible for the slow growth of the amount of circulating antibodies after primary infestation at a time when the ticks have already terminated their feeding, such as was the case of our experiments between days 10 and 20 after the first infestation.

Although a level of antibodies exceeding the level of the first natural infestation was reached in the blood of susceptible hosts by transfer of immunoglobulins from resistant pigeons, the immune resistance became manifest in recipients only in part (in two cases not at all), namely in those parameters which are closely related with the process of feeding on host (percentage of engorged larvae, period of feeding and weight of engorged larvae). The protracted effect, which became manifest in the duration of the premoulting period and the percentage of moulted larvae after secondary feeding on control and treated pigeons, was not recorded in larvae fed on Ig-recipients. This fact suggests that in the case of *A. polonicus* the host anti-tick immunoglobulins, ingested in the tick blood meal taken on resistant host, are quickly neutralized by the immune system of the tick (Ben Yakir et al. 1986) and therefore do not affect its further development, although the serum proteins have retained their antigenic properties in the hind gut for at least 20 days, as observed by Weitz and Buxton (1953) in *Ornithodoros moubata*.

The fact that in two Ig-recipients the resistance did not become evident (although in antibody levels and kinetics these pigeons did not differ from partially resistant host) points out the different reaction ability of different specimens. The development of a mere partial resistance in other pigeons after application of immunoglobulins from resistant hosts indicates the important role of other components of antibody-mediated, complement-dependent and cell-mediated effector mechanisms in the development of complete immune resistance to ticks.

The two-peak curve of the occurrence of anti-tick antibodies in infested Ig-recipients is evidently due to the detection of injected immunoglobulins at the first peak and host antibodies proper at the second peak, as follows from the time localization of these peaks until days 3 and 10 post infestation.

From 14 antigenic fractions recognized by serum of resistant pigeons in the homogenate from *A. polonicus* larvae only six (165, 45, 27, 23, 21 and 19 kdal) represent the

dominant antigenic fractions, which correspond with protein fractions of homogenate. Shapiro et al. (1986) found in the homogenate of salivary glands of *Rhipicephalus appendiculatus* 12 antigens recognized by serum of resistant guinea pigs from which only 5 were strongly recognized; these antigens had molecular weight of 94, 58, 35, 20 and 16 kdal. Only the 35, 20 and 16 kdal antigens were relatively constant, occurring also in nymphal and larval stages. The 90 kdal salivary gland protein of *R. appendiculatus* was strongly recognized by resistant rabbit serum as a main antigen, inducing partial host resistance to the feeding of this tick species (Shapiro et al. 1987). Brown et al. (1984) and Brown and Askenase (1986) demonstrated that a 20 kdal protein derived from salivary glands of *Amblyomma americanum* is the only antigen recognized by guinea pig anti-tick antibody. It induces skin reaction in tick-sensitized animals and protects naive animals from tick infestation. Whelen et al. (1984) and Wikel and Whelen (1986) found in *Dermacentor andersoni* larval antigens 4 components (98, 41, 35 and 25 kdal) which were recognized by guinea pig resistant serum.

Molecular weights of antigenic fractions from the homogenate of *A. polonicus* larvae (165, 45, 27, 23, 21 and 19 kdal) ascertained by us resemble the molecular weights of antigenic fractions of homogenates from other tick species and genera and it appears that the antigenic fractions of lower molecular weights are often the most antigenically active ones and play the decisive role in the antibody-mediated response of hosts.

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ОПОСРЕДОВАННЫЙ АНТИТЕЛАМИ ОТВЕТ ГОЛУБЕЙ НА КОРМЛЕНИЕ НА НИХ ЛИЧИНОК КЛЕЩА *ARGAS POLONICUS* И ХАРАКТЕРИСТИКА ЛИЧИНОЧНОГО АНТИГЕНА

Ф. Дусбабек, Ш. Лукеш, В. Матьха и Л. Грубгоффер

Резюме. Циркулирующие антитела против личиночного антигена клеща *Argas polonicus*, которые можно обнаружить в крови голубей с помощью ELISA, достигают самого высокого уровня через 3—6 дней после прикрепления личинок. Через 6—8 дней после их прикрепления, когда большинство личинок отваливается от хозяина, уровень антител в крови резко понижается, но через 10—15 дней вновь повышается. В ходе второго и дальнейших заражений динамика образования антител аналогична, но максимальные величины абсорбции при реакции ELISA при каждом дальнейшем кормлении личинок повышаются. Это находится в прямой корреляции с ростом иммунной устойчивости хозяев. Инъекции иммуноглобулинов устойчивых голубей вызывает у несенсибилизированных хозяев частичную устойчивость, проявляющуюся в статистически значимом ($P < 0,01$) понижении числа напитавшихся личинок, сокращении продолжительности кормления личинок и понижении их среднего веса после кормления. Уровень этой устойчивости не достигает параметров естественной устойчивости голубей во время второго заражения личинками клеща. Запаздывающее воздействие на продолжительность периода до линьки и процент линявших личинок, проявившееся у личинок после второго заражения, не выявилось у личинок, питавшихся на реципиентах иммуноглобулинов. Шесть крупных белковых компонентов из личиночного экстракта, с молекулярным весом приблизительно 19, 21, 23, 27, 45 и 165 килодальтонов, были идентифицированы сывороткой устойчивых голубей путем иммуноблотинга.

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F. D., Parazitologický ústav ČSAV,
Branišovská 31,
370 05 České Budějovice, ČSSR