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Relationship Between Spleen Weight Increase Factor (SWIF) of Mice and *Eperythrozoon coccoides.** (30376)

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A transmissible spleen weight increase factor (SWIF) encountered during the transmission of a neoplastic disease in mice was previously described(1). The possibility was considered at that time that the SWIF could be *Eperythrozoon coccoides,* since morphological elements said to be characteristic of *E. coccoides* were, under some circumstances, observed in blood smears of mice infected with the SWIF. Under other circumstances, blood of infected mice did not disclose these elements. Therefore, some reservation was expressed concerning the identity of the SWIF with *E. coccoides.* We now present data which quantitatively relate spleen weight increase to certain of these characteristic morphological elements in the blood and, hence, to *E. coccoides.*

Materials and methods. The preparation of infectious material and the mice used were previously described. When infectious plasma was desired, blood was collected by the orbital method(2) with the aid of heparinized micro blood collecting tubes.‡ After centrifugation at 800 × *g* for 15 minutes in the cold, the plasma was collected and, unless immediately used, stored in the CO_2 deep freeze.

The average response of 5 mice at 7 days determined a single experimental point. The body weight of each mouse was determined at sacrifice, its spleen excised, and the spleen weight (mg) per gram body weight determined. The mean of the 5 values and its standard error were calculated.

In many instances the determination of

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TABLE I. Effect of Cortisone on Spleen Weight and Infectivity of Plasma.

Treatment	Spleen wt/body wt, mg/g	Infectivity of plasma, log SED_{50}/0.1 ml
None	6.24 ± 1.03	—
Infected	16.0 ± 1.1	6.3
" , cortisone*	9.9 ± .4	6.5

* 1 mg of cortisone acetate was inoculated subcutaneously on day of infection and on the 2 succeeding days.

response as indicated above was adequate to assess the effect of various treatments on the activity of the SWIF. When more accurate estimates were required, the procedure of Reed and Muench for viruses(3) was adapted to determine the "Spleen Enlarging Dose 50%" or SED_{50} of the SWIF. This represents the infectious unit and is defined as the highest dilution which, when inoculated intraperitoneally into susceptible mice, will produce a spleen weight of at least 10 mg/g body weight in 7 days in 50% of the recipient mice.

Phosphate buffered saline, pH 7.4, containing 0.5% bovine serum albumin was routinely used as a diluent in titrations. Infectious material was maintained in an ice bath until use.

Splenectomy was accomplished under anaesthesia with ether. Mice were infected 24 hours after splenectomy.

For examination of morphological elements, blood films were air dried and immersed in methanol for 3 minutes. The dried fixed smear was covered for 30 minutes with a solution of Giemsa stain prepared by addition of one drop of commercial "Giemsa Stain Solution"§ to 1 ml of distilled water. The stained film was rinsed under a flow of distilled water and dried. Microscopic examination was made under oil with a 97× ocular and 10× eye piece.

Results. Role of the spleen in the infectious process. It was observed that spleens were only slightly enlarged in infected mice treated with cortisone, although their plasmas were as high in infectivity as in untreated mice (Table I). It seemed, therefore, that the spleen might not play an essential role in the propagation of the agent. To test this, the infectivity of plasma of infected splenectomized mice was compared to that of sham-operated mice. The results are summarized in Fig. 1. It is evident that on the fourth day after infection, the plasma of splenectomized mice was about one hundred times as infective as that of intact mice, indicating the spleen was, in fact, a deterrent to the infectious activity of the plasma. Although not shown here, maximum spleen weight and plasma activity coincided at 7 days in intact mice.

Morphological elements in blood of infected mice. Attempts to observe Giemsa-stained morphological elements, said to be characteristic of *E. coccoides,* which the SWIF resembled, in blood films of infected animals, have been discouraging. Occasional samples showed a few annular elements, or rods and coccoidal forms, almost at the limits of visibility of the light microscope, even when the plasma had a titre as high as 10^7 SED_{50} per ml. The substantial increase in the titre of the plasma of infected, splenectomized animals encouraged a more intensive investigation of Giemsa-stained blood films of such mice.

Blood films were examined beginning at

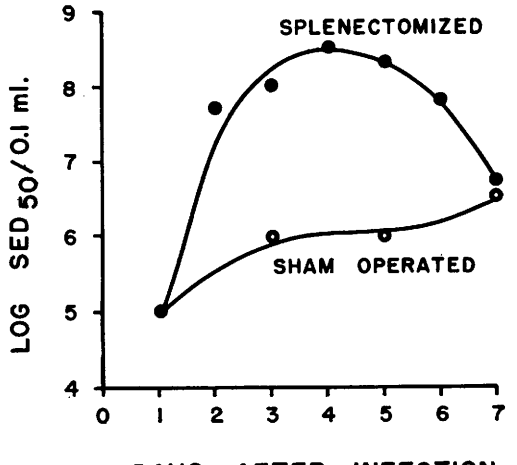

FIG. 1. Effect of splenectomy on titer of *E. coccoides* in plasma. Mice were splenectomized or sham-operated 24 hr prior to infection. After the indicated times, plasma was collected and titrated in normal mice.

§ National Aniline Division, Allied Chemical Corp., New York.

TABLE II. Relationship Between Annular Elements in Blood Films and Infectivity of Plasma.*

Day post-inoculation	Total intracellular particles per 0.1 ml blood	Total extracellular particles per 0.1 ml blood	SED_{50} units per 0.1 ml plasma	Extracellular particles/infectious units
4	1.85×10^8	6.67×10^8	3.2×10^8	4†
6	1.95×10^8	1.48×10^8	$.6 \times 10^8$	5†

* Intra- and extracellular annular particles per 1000 erythrocytes were counted in Giemsa-stained blood films. Independent blood counts (erythrocytes per 0.1 ml blood) permitted the results to be expressed in particles per 0.1 ml blood.

† Calculated on the basis that plasma represents approximately 50% of the volume of whole blood.

one day post-infection. On the first, second and third day, few morphological elements characteristic of *E. coccoides* were seen. The elements which did appear were annular and were either attached to erythrocytes or free in the plasma. On the fourth day, the appearance of stained blood films was drastically changed. There was a large increase in the number of intra- and extracellular elements, the majority of the latter consisting of purplish, rather uniform rings, approximately 1/20 the size of an erythrocyte (Fig. 2). The intracellular elements occurred mainly in polychromatic erythrocytes, and were heterogenous in size and structure. They consisted of plain and signet rings, as well as of coccoid forms. Fig. 2 (D) depicts the position occupied by certain of these elements in an erythrocyte which then evidently disintegrated. An occasional polychromatic erythrocyte appeared to be ruptured at one end, the elements seemingly streaming out.

On the 6th day, the number of extracellular elements was approximately 10% of that on day 4, although the number of infected polychromatic erythrocytes was about the same. On succeeding days, very few elements were seen, either intra- or extracellularly.

Relationship between annular elements and infectivity of plasma. The availability of a quantitative method for determining infectivity, and the observation that a significant number of microscopic annular elements occurred in infected, splenectomized mice beginning on the fourth day of infection, made feasible an attempt to relate infectivity to the particles observed in the blood films.

Table II summarizes an experiment which permitted a relationship to be made between number of annular particles and number of infectious units in plasma. The number of annular elements within polychromatic erythrocytes was fairly constant at the intervals studied. The number of extracellular elements, however, was much greater at 4 than at 6 days and corresponded to the titre of the plasma. Thus, it was deduced that these elements represented the infectious particles of the plasma, and it was calculated that an infectious unit (one SED_{50}) consisted of 4 to 5 such particles. It should be mentioned that whole blood had approximately one log higher titre than plasma, indicating that certain cell-associated elements were also infectious. The nature of these was not investigated but, as already indicated, rings also occurred intracellularly.

Discussion. Since the discovery of *Eperythrozoon coccoides* by Schilling(4), who named it, and independently by Dinger(5), knowledge of the biology of this ubiquitous but relatively little known organism has advanced slowly. Its taxonomy is still uncertain, though it is presently classified(6) among the Protophyta (Order, Rickettsiales; Family, Bartonellaceae). Infection with this organism has been recognized almost entirely by the appearance of a variety of near-submicroscopic structures in the blood of infected animals, particularly after splenectomy but also, somewhat unpredictably, under other circumstances, such as in some tumor-bearing or very young mice. Splenomegaly has been mentioned as a sequela of infection with *E. coccoides* but, until now, no relationship had actually been established between any of the morphological structures in plasma and splenomegaly. There was, in fact, doubt as to which of the particles, of the variety observed, were infectious. Our data,

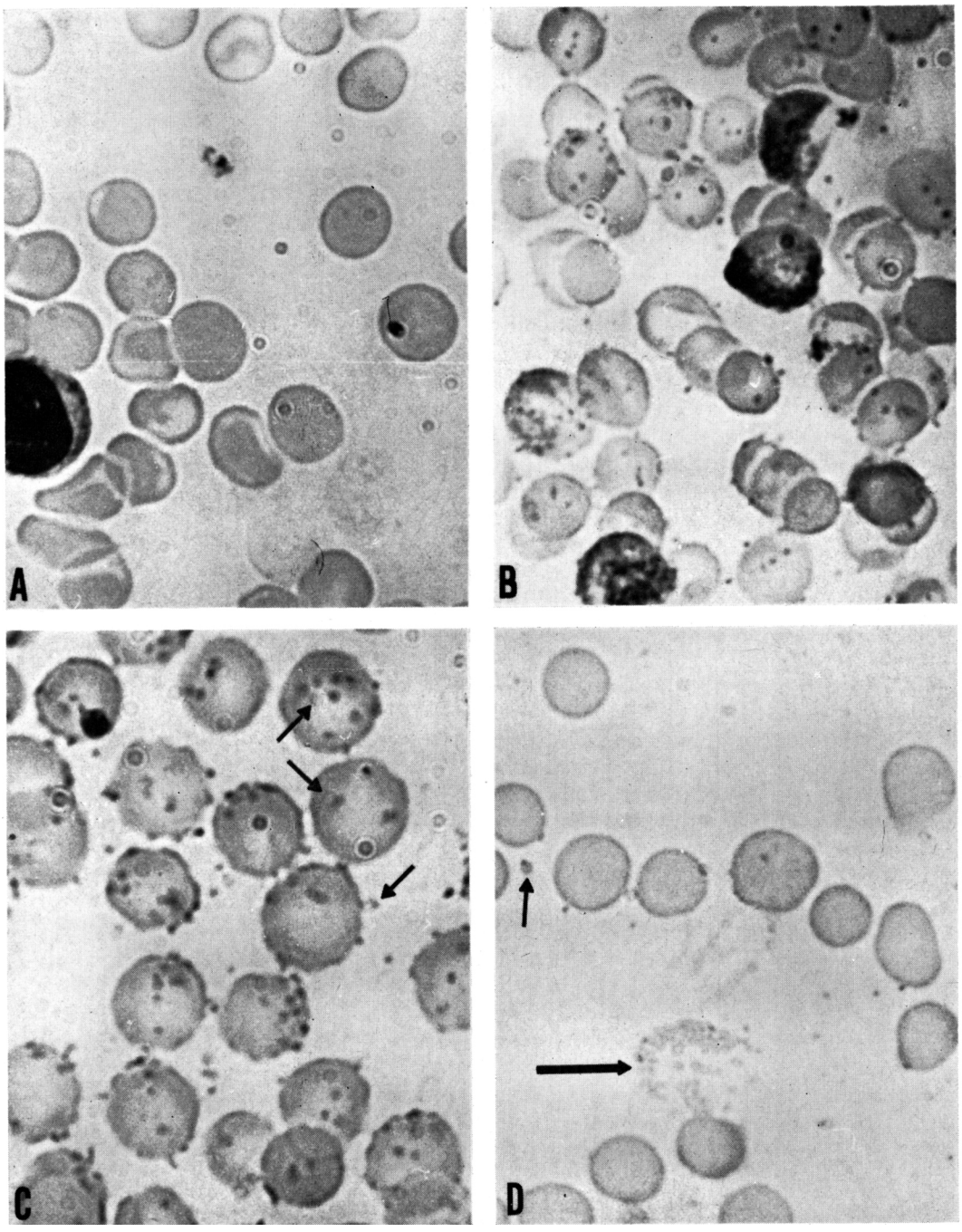

FIG. 2. Giemsa-stained blood smears from mice infected with *E. coccoides* 1 day following splenectomy. Magnification × 1500.

A. 4 days post infection. A field of fairly uniform rings, mostly extracellular, is shown.

B. 4 days post infection. Intracellular and extracellular rings and coccoid forms are demonstrated.

C. 5 days post infection, showing rings, signet rings (arrows) and coccoid forms.

D. 4 days post infection. Large arrow points to a structure consisting of many rings arranged in a way suggesting that they occupied an erythrocyte which degenerated. Smaller arrow points to a signet ring.

showing that a quantitative relation exists between the extracellular elements of blood of splenectomized mice and the infectivity of its plasma (determined by spleen weight increase in intact mice), permit the conclusion that these annular elements were the infectious particles responsible for the splenomegaly in intact mice. Since these annular particles are one of the most prominent of the morphological structures designated *Eperythrozoon coccoides,* it follows that the spleen weight increase factor (or SWIF) is identical with *E. coccoides.* Our estimate that 4 to 5 annular elements comprised an infectious unit indicates that *E. coccoides* is an agent of exceptionally high infectivity. Before appreciable numbers of characteristic rings were seen in blood smears, it was necessary for the titre of the plasma to increase from about 10^7 to 10^9 SED_{50} per ml. This increase in titre occurred only in splenectomized mice, and indicated that the spleen was a deterrent to the infectivity of the plasma.

It was previously emphasized(1) that the SWIF (*i.e., E. coccoides*) was tenaciously associated with the lactic dehydrogenase (LDH) elevating agent. Separation of the LDH virus from this association was accomplished by differential filtration, or by treatment of infected mice with a tetracycline antibiotic, which eliminated *E. coccoides* but did not affect the LDH agent. Furthermore, it was demonstrated that when freed from *E. coccoides,* the LDH agent did not induce splenomegaly. Riley(7) has also found the LDH agent to be non-splenomegalogenic, and has additionally reported(8) obtaining *E. coccoides* free from the LDH agent by passage of both through rats. More recently(9) Riley has shown that the LDH virus, while not significantly increasing the size of the spleen, does synergize the effect of *E. coccoides* in this regard. Our data presumably reflect such synergism since the infectious material available to us contained both agents. The observed spleen weights may, therefore, have been exaggerated, but this does not affect the relationship we have established between the extracellular particles and spleen weight. Indeed, the net result of the presence of both infectious agents would be to increase the sensitivity of the assay for *E. coccoides.*

Aside from its intrinsic interest as a rather unusual, though ubiquitous, infectious entity, knowledge of *E. coccoides* is essential to investigators of neoplastic diseases and infectious agents in the mouse. It is frequently present in transplantable tumors(10) and may, in part, be responsible for the splenomegaly so widely commented upon as a sequela of transplantation of such tumors(11). The pronounced synergistic effect of *E. coccoides* with a variety of other infectious agents, oncogenic and non-oncogenic, is now well known(11).

Summary. A quantitative assay was devised for the spleen weight increase factor (SWIF). With it, a precise relationship was established between extracellular annular structures in the blood of infected splenectomized mice and the spleen weight of intact mice. Since these annular structures are prominent morphological elements of the entity designated *E. coccoides,* it was concluded that the SWIF is identical with *E. coccoides.* It was found that the spleen *in situ* is a deterrent to the infectivity of the plasma.

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