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Host-Parasite Relationships with *Brucella neotomae*

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ABSTRACT

GIBBY, IRVIN W. (Cornell University Medical College, New York, N.Y.), AND ANNA M. GIBBY. Host-parasite relationships with *Brucella neotomae*. J. Bacteriol. **89**:9–16. 1965.—An investigation was undertaken to ascertain whether a vigorous parasitic state could be evolved by transfer procedures in the host-parasite system, *Brucella neotomae* versus the white mouse. Visible disease was not maintained and initial high doses were abruptly decreased if the parasite was serially transferred through host animals without the use of an adjuvant. Of several adjuvants tested, 5% mucin was the most effective in enhancing *Brucella* infections in the white mouse, and with this adjuvant a vigorous lethal disease was maintained in serial transfer. In one transfer series, bacterial colonies of a form different from *B. neotomae* were obtained. Substrains of the altered bacterial form were agglutinated by *Brucella* antiserum and exhibited other properties consistent with the genus, *Brucella*. Cultures and selected clones of this organism in low doses caused rapidly lethal disease in the white mouse in the absence of mucin. Also, the disease could be maintained in serial transfer as an acute lethal process without mucin. It is concluded that the host-parasite interaction was drastically altered with the emergence of a highly virulent parasite in an infectious state that had previously been relatively benign. Various related aspects of parasitism are discussed.

It appears reasonable to attribute the virulence properties of pathogenic microorganisms to genetic determinants in much the same manner as are other microbial characteristics, e.g., resistance to antibiotics. If this assumption is correct, it may be expected that mutant pathogens with high, low, and intermediate virulence properties arise in a population of microbial parasites. The selection of these various candidate mutants is dictated by the survival value of the virulence property, as tested in the continuing host-parasite interaction. Documentation to be presented illustrates that many disease entities evolve toward lessened overt pathology, and this decrease appears to result, in part, from diminished parasite virulence. However, there is no *a priori* reason, implicit within the microbe, for the lack of a potential for a very broad spectrum of virulence effects, including very high virulence levels; and evolution toward high, low, or intermediate virulence levels would each be theoretically possible, provided that survival of the parasite (in a surviving host species) would be optimized. It follows that microbes may be potentially capable of markedly greater pathogenicity than is commonly recorded for wild strains.

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Scant attention has been given to the exploration of the total potential of the parasitic state. Thus, the conditions and mechanisms in host-parasite interactions that determine attenuation, stability, or intensification of particular disease entities are largely undefined. The objective of this investigation is to examine the theory that a mutant microbial parasite with markedly enhanced virulence can be encountered and isolated from a benign infectious state by suitable manipulations of the host-parasite interaction. Manipulation in this sense is analogous to screening procedures used to isolate antibiotic-resistant mutants, in that an environment is sought that selects for the mutant of interest.

Experimental brucellosis in the white mouse was chosen as the host-parasite complex for this investigation. In this disease, infected mice retain a relatively high census of the organism over a period of weeks with little visible deleterious effect, and lethalities are encountered only with very high doses of the organism.

MATERIALS AND METHODS

Bacterial culture. N. B. McCullough kindly supplied a culture (no strain designation) of *Brucella neotomae* (Stoenner and Lackman, 1957) for use in this investigation. As received, it was a smooth strain and in all input work smooth colonial form

was maintained. Stock and working cultures were grown on Trypticase Soy Agar (TSA) slants with incubation restricted to the overnight period (16 to 17 hr) to avoid cultures that were beyond the active growth phase.

Experimental animals. Specific pathogen-free Swiss Webster mice were used as host animals.

Procedures for infecting animals. To initiate serial transfer procedures, 0.5 ml of a saline suspension of bacteria was injected intraperitoneally into each mouse in a group. A similar procedure with appropriate dilutions of the initial suspension was used for titration of lethal effects.

For mouse-to-mouse transfers, two mice of a prior group were killed, as required by the protocol, or, in a few instances, mice that died while under close surveillance were used as source materials for serial transfer work. Livers and spleens were ground in 10 ml of sucrose buffer solution (Smith et al., 1961) contained in the 25-ml flask of an MSE tissue grinder (Instrumentation Associates item 5045, manufactured by Manor Royal, Crawley, Sussex, England). Tissue debris was discarded after slow centrifugation. After additional centrifugation at 5,000 rev/min (3,300 × *g*) for 70 min, the sediment was washed-up in a small volume (usually 3 ml) of saline (or 5% mucin, see below), and this suspension was used as source material for inoculation into the next group of mice. Thus, infective material was passed directly from mouse to mouse with accompanying, but not intervening, culture work. In all cases, the census of *Brucella* organisms in suspensions used as inocula was ascertained by a standard counting method in which cultures or homogenized tissue sources were diluted and surface-streaked on plating media.

Media. TSA, with the *p*H adjusted to 7.1 prior to autoclaving, was the standard medium used for slants and plates in this work. Sterile dextrose to 1% (w/v) was added aseptically after autoclaving.

As a later requirement, an enriched medium, referred to as TSAB, was provided by adding 0.2% whole defibrinated rabbit blood and 0.5% rabbit serum to the TSA medium.

Adjuvants. Mesoerythritol, cortisone, liquoid (polyanethol sodium sulfonate), and mucin were administered to separate groups of mice in conjunction with various transfer or challenge procedures. Of these, only mucin had a virulence-enhancing effect. Bacterial suspensions or tissue sources of bacteria were mixed with sterile 5% mucin, and 0.5 ml of this suspension was given, intraperitoneally, to each mouse.

Identification of variant strains of Brucella. Microscopic examination and slide agglutinations served as screening techniques for new colony types as they appeared. Tube agglutination tests for the examination of variant strains followed the procedures of Spink (1956). Antigens were prepared as saline suspensions of bacterial cells. In some instances, the cell suspension was heated to 60 C for 1 hr, or suspensions that were not homogeneously dispersed were ground in the MSE tissue grinder. Difco anti-*B. abortus* serum was used throughout.

Motility, catalase production, H_2S production, and nitrate reduction were determined for various strains, as required.

RESULTS

Pathogenicity of parent strain. Data on mortality response versus dose for *B. neotomae* infections in white mice are given for two experiments in Table 1. An estimate from these data would place the LD_{50} value at approximately 2 billion cells.

Serial passages of *B. neotomae* in the white mouse were performed without the use of adjuvants in a number of experiments. Composite results indicate that *B. neotomae* infections of white mice could not be sustained by mouse-to-mouse transfers. The dose available from homogenates of livers and spleens for transfer to each succeeding serial group was less than one-tenth of the dose that was given to the immediately preceding group. The failure to maintain a significant degree of illness during serial transfer was not influenced by moderate differences in initiating dose or by transfer schedules.

Enhancement with mucin of pathogenicity of parent strain. The use of 5% mucin as a suspending material for inocula, either from initial cultures or from tissue homogenates, drastically altered the infectious process so that 100% mortality was observed in all groups of mice as long as serial transfer was maintained. The disease process with mucin produced marked illness by the second day, with deaths occurring predominantly on the second and third day after inoculation. With this system, dose levels of the infecting organism were maintained at approximately 10^9 cells for successive transfer

TABLE 1. *Response of white mice inoculated with varying doses of Brucella neotomae*

Expt	Dose* (cells)	Deaths per no. of animals in group
1	3.93×10^9	6/6
	3.93×10^8	0/6
	3.93×10^7	0/6
2	8.0×10^9	6/6
	4.52×10^9	6/6
	2.54×10^9	4/6
	1.43×10^9	0/6
	8.0×10^8	0/6
	4.52×10^8	0/6

* In all cases the dose is in terms of living (culturable) organisms. Effects of equivalent doses of nonliving organisms were not measured.

intervals. The maintenance of high mortality and vigorous parasite multiplication in transfers with mucin contrasted sharply with the results obtained without the use of an adjuvant. Control animals were not noticeably affected by the administration of sterile 5% mucin. [Substitution of heparin for mucin, as suggested by Lambert and Richley (1952) and Smith (1953), was not undertaken.]

Detection of mutant strain in serial transfers. Experiments were undertaken to serially transfer the agent through the host, utilizing mucin as an adjuvant. In work of this type, the probability of encountering a mutant with altered parasitic properties is low, and numerous transfer experiments that were negative in outcome are omitted. Essential data on one experiment of interest are shown in Fig. 1. While not illustrated, experiments of this type were conducted with two groups of mice at each transfer interval. Each group in the primary series received inoculum in mucin while an "indicator" group received inoculum in saline, to register the possible occurrence of an infectious process that was independent of mucin. Of the animals in group 1, mucin-treated mice, one had died and two were killed on the third day. On the fifth day, one of the two remaining mice had died, and one very ill mouse was used for inoculum to initiate group 2 of the illustrated series. As the transfers proceeded in the sequential groups of mucin-treated mice, high mortality was observed in a number of respective indicator (nonmucin) groups of mice, and it was possible to maintain the infection in direct transfers in nonmucin mice, beginning with group 4 animals. Subsequently, there were ten additional transfers in nonmucin mice, and, in each, high mortality was observed. Data to be presented indicate that an altered form of brucellosis had been encountered.

Parallel with the animal transfer work described above, cultures of tissue homogenates were examined at each transfer interval. Colonies distinctly different from the parent form were cultured from homogenized tissues of group 3 mice. In the transfer from group 3 to group 4, the parent colony type was present at a concentration of 3×10^7 cells per ml of transfer material, and it was estimated that the new colony type was present in about equal concentration. In succeeding animal transfers, the new colony type rapidly supplanted the parent type.

The new colony type in plates from the group 3 transfer appeared as a semitransparent blue-gray colony. Microscopic examination of Gram-stained smears revealed cocco bacillary forms typical of *Brucella*. Suspensions of the organism

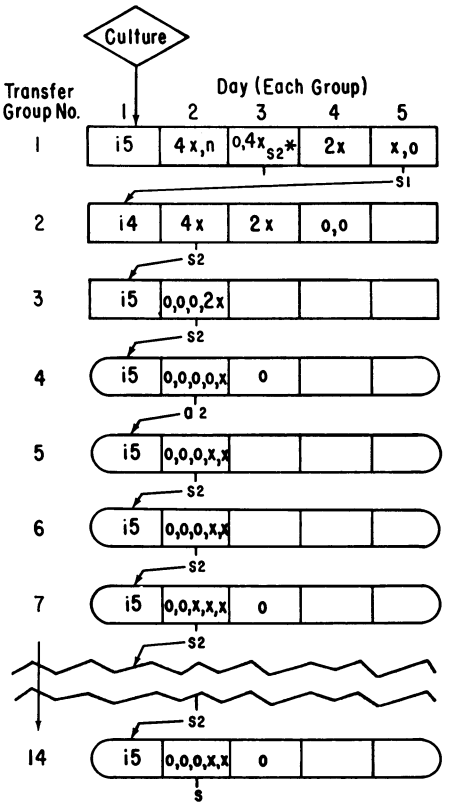

FIG. 1. *Transfers of Brucella neotomae in mice.*

yielded positive slide agglutination with Difco anti-*B. abortus* serum. [*B. neotomae* and *B. abortus* strains yield essentially identical serological reactions (Stoenner and Lackman, 1957).] For ease of description hereafter, the new colony variant will be referred to as strain ND (neotomae derivative); substrains to be described will be numbered ND-1, ND-6, etc.

Enriched culture media for variant strains. The growth pattern of strain ND on TSA indicated that multiplication of the organism would occur only in specimens which contained animal tissue fractions that were not highly diluted. Accordingly, TSAB medium was utilized. The medium was essentially transparent and supported quantitative growth and continuous subculture of strain ND.

Isolation of clones from strain ND. A number of

colony types were observed in ND cultures obtained from animal sources. Various isolations were made, and the infectious properties of several substrains were examined.

A dominant colony type, labeled ND-4, was readily isolated. This colony was stable in colonial form, except for the appearance of a pink-orange subcolony or a whitish semitransparent subcolony, both relatively rare events. Colonies of strain ND-4 could be readily observed with low-power magnification after 24 hr of growth. With oblique light, the colony appeared smooth and translucent with a gray-green to blue coloration. At 48 hr, with oblique light, ND-4 colonies appeared somewhat flattened with an irregularly colored central zone with green, gray, and pink coloration. A peripheral zone appeared gray with a pinkish cast that was more evident in confluent areas. A slightly raised central zone could be seen in many colonies.

A substrain characterized by cloudy gray colonies with a pink cast, a similar substrain with colonies that had a definite whitish appearance, and a third type that produced colonies with an even gray, semitransparent appearance were labeled ND-6P, ND-6W, and ND-6G, respectively. Other mutants were seen in the course of culture work with strain ND-6: in form and color, the colonies of one of these strongly resembled colonies of *B. abortus*; one was a smooth opaque white colony. Both were stable in subculture and gave positive slide agglutination tests with anti-*B. abortus* serum. Neither produced pathology that was typical of strain ND.

Identification of derived cultures. The various substrains of culture ND had cellular morphology typical of *Brucella*. For substrains ND-4, ND-6W, ND-6G, and ND-6P, the results of various cultural tests were identical as follows: catalase-positive, nitrate reduced to nitrite, negative for motility, H_2S not produced or produced only in trace amounts, acid (< *p*H 6.0) produced on dextrose-containing media. The parent *B. neotomae* strain yielded the same results, except that H_2S was produced by 48-hr slant cultures. The vigorous acid-producing capability of *B. neotomae* has been previously noted (Gibby and Gibby, 1964). Substrains of ND were not examined with media containing thionin or basic fuchsin.

Positive slide agglutination tests were observed regularly with suspensions made from ND cultures. Usually, the precipitate formed with ND cultures was finer than that observed with *B. neotomae*, but the progressive formation of the precipitate could be observed and the contrast with control suspensions was definite. In tube agglutination tests with homogenized antigens, substrains ND-6G, ND-6W, and ND-6P yielded positive reactions through the 1:400 dilution, with the latter strain having the most vigorous reaction at all dilutions.

The results given for these investigations lead to the conclusion that the ND strains were, in fact, members of the genus *Brucella*.

Serial transfer with substrain ND-4. A transfer experiment was conducted with culture ND-4 to establish whether the organism would produce severe pathological effects after isolation and in vitro propagation. The organism was passed from mouse to mouse through seven serial passages when the experiment was terminated. No mucin was used in any transfer operation. Mortality was 100% in each group of mice. Data on the dose at various intervals indicate that the parasite multiplied sufficiently to maintain the passage dose at a high level. Cultural examinations showed that pure cultures of ND-4 were obtained at each interval, and that colonial form was constant throughout the experiment. (Rarely, a pink mutant colony was seen. Its numbers were extremely small in relation to the total count of ND-4. Occurrence of the pink colony did not increase in frequency as the experiment progressed.) Thus, the in vitro-propagated substrain ND-4 was capable of reproducing a disease picture entirely similar to that seen in prior experimentation, in which the ND strain had not been subjected to in vitro propagation.

Titration of mortality effects of several ND substrains in white mice. After strain ND-6 had been cultured in vitro and before it had been separated into pure clones, it was assessed for mortality effects in white mice. Similarly, as they became available, clones ND-6P, ND-6W, and ND-6G were also assessed for the capacity to cause lethal infections (Table 2). The heterogeneous culture ND-6 produced a lethality rate of 100% at all dose levels, including the lowest measured dose of approximately 2,000 cells. Of clones derived from ND-6, the most vigorously pathogenic was ND-6G. This strain produced lethal rates equal to that of the heterogeneous culture, while strains ND-6P and ND-6W caused markedly less lethality than either the mixed ND-6 culture or the pure gray clone, ND-6G. The capacity of the ND-6G strain to cause 100% lethal responses in white mice at dose levels of 2,000 organisms is in marked contrast to the lethal capability of the parent *B. neotomae* strain, as described in an earlier section. It may be recalled that the LD_{50} of the parent strain of *B. neotomae* was estimated to be about 2 billion cells, whereas with strain ND-6G no LD_{50} end point was defined even at the lowest dose employed.

TABLE 2. *Response of white mice inoculated with varying doses of a derived culture of Brucella neotomae*

Substrain*	Dose (cells)	Deaths per no. of animals in group
ND-6	2.08×10^7	3/3
	2.08×10^6	3/3
	2.08×10^5	3/3
	2.08×10^4	3/3
	2.08×10^3	3/3
ND-6-G	3.18×10^7	3/3
	3.18×10^6	3/3
	3.18×10^5	3/3
	3.18×10^4	3/3
	3.18×10^3	3/3
ND-6-P	1.2×10^8	1/3
	1.2×10^7	0/3
	1.2×10^6	0/3
	1.2×10^5	0/3
	1.2×10^4	0/3
ND-6-W	1.61×10^8	1/3
	1.61×10^7	1/3
	1.61×10^6	1/3
	1.61×10^5	0/3
	1.61×10^4	0/3

* ND-6 = neotomae derivative culture #6 (not separated by colony type). Colony types: ND-6G(ray), ND-6P(ink), ND-6W(hite).

DISCUSSION

The continued existence of a parasite within an ecological community generally tends to evolve host-parasite interactions that are characterized by diminished pathology (Simon, 1960; Dubos, 1958; Burnet, 1953; Swellengrebel, 1939; Zinsser, 1943; Fenner, 1959).

Basic mechanisms that may, in part, apply to this trend toward lessened pathology are identifiable in Fenner's (1959) account of myxomatosis in Australian rabbits. This work is particularly valuable in that the whole record of a host-parasite interaction is available from the time of initiation of the disease as a highly destructive process until a state of decreased pathology was clearly measurable. In this episode, the prolonged challenge of a highly destructive disease selectively removed susceptible host animals, with the result that a more resistant host population emerged. Also, highly virulent virus was differentially removed from the host-parasite interaction because host death outpaced transmission. These alterations in host and parasite account for, and illustrate succinctly, a trend toward lessened pathology in a continuing disease of animals in the wild state.

Simon (1960) cited examples of increasing mortality and progressively accelerating disease processes in epidemics that provided opportunity for rapid contagion in host animals. There must be, however, some limit beyond which virulence cannot progress in natural disease states, for if virulence became extreme a threshold level would be encountered that would preclude transmission prior to host death, and the parasite would pass out of existence because it would be "'¬o virulent" for species survival. Suggestive of this circumstance is a report by Pollitzer and Li (1943), who observed that the course of disease rapidly increased during the progression of an epidemic of plague and that contagion failed when host death became extremely rapid.

It is possible, therefore, to postulate that, in natural disease states, the expression of virulence in microorganisms is limited by two general mechanisms: (i) the interaction tends to reduce pathology by adjustments in virulence of the parasite and resistance of the host, and (ii) the evolution of parasites with very high virulence has an upper boundary which is fixed by capacity for contagion and transfer opportunity.

These limiting mechanisms may be entirely obviated in experimental work. In fact, extreme virulence, as a parasite property, should be possible, provided conditions in a manipulated host-parasite interaction are in consonance with the emergence and maintenance of this property. This investigation attempts a preliminary inquiry concerning the possible range of virulence in the absence of restraints imposed by natural disease.

It was shown in the prior section that *Brucella* infections in white mice resulted in transient illness and insufficient multiplication of the parasite to maintain the infection in mouse-to-mouse transfers. The use of mucin as an adjuvant in such infections, however, resulted in lethal infections and permitted in vivo multiplication of the parasite at a sufficiently high level to maintain an acute, lethal infectious process in sequential mouse-to-mouse transfers. A number of such passage experiments were conducted, and in one of these an altered disease pattern was observed. In this altered form, *Brucella* infections of mice were lethal without mucin or other adjuvants. Reisolated cultures that required blood enrichment for in vitro growth were identified as altered strains of *Brucella*. These were more than one million times more lethal for mice than was the parent strain.

These findings indicate that the trend toward diminished pathology observed in natural disease processes can be reversed experimentally. The conditions imposed on the experimental system include the necessity for in vivo parasite multiplication and an opportunity for effective transmission. Whether these conditions are achieved

in natural disease, to permit the emergence of a virulent pathogen from a previously quiescent disease state, is problematical. An eruption of abrupt and fatal psittacosis in captive birds, as described by Burnet and Macnamara (1936), may illustrate the first condition; i.e., degradation of host defenses probably permitted parasite multiplication that have previously been restrained. Except, however, for this shift in the immediate equilibrium between host and parasite, the incident probably does not represent the emergence of a parasite with markedly altered properties. Evans (1960) indicated that naturally occurring differences in the virulence of poliomyelitis virus have not been correlated consistently with high or low "epidemiologic virulence." Owen et al. (1961) failed to find alterations in virulence in two naturally occurring epizootics of tularemia. Wilson and Miles (1955) took the view that "the evolution within any parasitic species of a strain of high epidemicity or virulence is an occasional event, rather than part of a normal or periodic process . . . "

Although the mechanisms of host-parasite interaction that appeared to dominate the myxomatosis episode probably have considerable influence in other host-parasite encounters, additional factors and mechanisms are required for a comprehensive theoretical pattern that may be applied generally to the parasitic state.

Dubos (1958) has indicated that populations previously unexposed to a disease may show a high degree of susceptibility. Heagerty's (1928) vivid description of smallpox in the American Indian in colonial times leaves little doubt that host susceptibility was extreme in continued epidemic episodes. Hutt's (1958) presentation of the history of an epidemic disease of "the oysters of Malpeque Bay" supports the view that a population may initially be highly susceptible to parasitic attack but may develop, during several generations, essentially complete genetic resistance to the pathological effects of the parasite. On the other hand, Evans (1960) provides a sound precautionary attitude toward regarding the "virginity" of previously unexposed populations as the controlling reason for the extensive pathology that often follows the initial introduction of a disease. His analysis of the experience of the Fijians with measles, in 1875 and subsequently, contradicts the generality that severe fatal disease episodes select host population elements that are genetically resistant to specific disease entities.

The history of syphilis in European peoples since 1495 illustrates a marked trend toward decreased pathology in a sustained host-parasite interaction (Zinsser, 1943; Winslow, 1943). However, it seems unlikely that fatalities or destructive pathology in this disease would have been extensive enough to differentially eliminate susceptible host-breeding stock. Also, the differential elimination of highly virulent strains of the microbe by rapid host death appears not to be an important factor in the diminished pathology of the disease. Thus, the shift toward mildness in this instance must imply the influence of additional factors.

The effect of prior ecological history upon parasite properties may be illustrated by tularemia in man. In America, an overall mortality rate of 7.4% was recorded prior to the advent of streptomycin (Francis, 1948). Jellison and Parker (1945) have presented convincing data for associating the bulk of human cases in North America with the geographical distribution of the cottontail rabbit. In Soviet Russia, with other rodents as propagating hosts, the human mortality from tularemia, in the absence of antibiotic therapy, has been recorded to be approximately 1% (Glass, 1948), and direct comparisons of American and Russian strains of *Francisella tularensis* (*Pasteurella tularensis*) (Olsufiev, Emelyanova, and Dunayeva, 1959) have shown that Russian strains have lower virulence for experimental animals (Olsufiev et al., 1959).

Effects of host resistance upon the host-parasite interaction have been studied by Wellhausen (1937) and Lincoln (1940). These workers showed that *Phytomonas stewartii* (bacterial agent of maize wilt) increased in virulence on passage through resistant host plants while a decrease in virulence was obtained when passage was accomplished with susceptible host plants. In both of these investigations, cultures intervened between successive hosts, i.e., host → culture → host, etc., and the degree to which the transfers represent natural epidemiological phenomena is obscure. The role of host resistance as described by these authors receives some substantiation from Fenner (1959), who pointed out that an increase in host resistance may offer selective advantage to a parasite with increased virulence, because the disease process must be of sufficient vigor to be capable of shedding the microorganism into the interhost channel for effective transmission. Zelle (1942), working with *Salmonella typhimurium* in mice, was unable to assign a role to host resistance but observed increased virulence of the parasite with animal passage, whether the host was resistant or susceptible.

Silverman, Drawdy, and Kautter (1963)

did not observe virulence changes in *Listeria monocytogenes* during passage, by several routes, in monkeys and in mice. Owen et al. (1961) have reported that a number of types of serial passage experiments failed to cause alteration in the virulence of *F. tularensis*. In the interpretation given by the latter authors to their investigations, the effect of serial passage, either in natural epizootics or in experimental disease, has no clear role in alteration of virulence properties of the parasite. Findings given in the cited work are in opposition to a number of reports (Green and Wade, 1929; Dieter and Rhodes, 1926; Green, 1943; and Philips, 1935) which indicate that serial passage resulted in marked enhancement of the virulence of wild strains of *F. tularensis*.

It would appear that individual mechanisms that may be clear-cut in a particular host-parasite interaction may not have a clearly defined influence in other situations, and that the number and type of mechanisms that apply generally to the parasitic state may obfuscate easy generalizations.

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