come appreciable, permitting temperature gradients with resulting thermal convection, using small or electrically charged particles so that diffusion or interparticle attraction can occur, employing non-Newtonian fluids, applying an external electric field, and so forth. See R. S. Allan and S. G. Mason, *Proc. Roy. Soc. Ser. A* **267**, 62 (1962); A. Karnis and S. G. Mason, *Trans. Soc. Rheol.* **10**, 571 (1966); F. Gauthier, H. L. Goldsmith, S. G. Mason, *Rheol. Acta* **10**, 344 (1971); E. B. Vadas, H. L. Goldsmith, S. G. Mason, *J. Colloid Interface Sci.*, in press.

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7. Many modes of perturbation can be considered. A simple one is to perturb all particles having identical ϕ_1 at a particular t/T (± 9 in the example) in the direction of the equilibrium distribution of orientations for each value of ϕ_1, by using a Gaussian distribution of perturbations of the phase angles κ given by $\tan \phi_1 = r_e(\tan 2\pi t/T + \kappa)$. This corresponds to identical bursts of rotary diffusion at each reversal. The values of M_1 in Fig. 1 are calculated (*6*) for perturbations of κ with a standard deviation of 0.2 radian at each reversal.
8. Supported by grant MT-4012 from the Medical Research Council of Canada.

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Antibody to HSV-2 Induced Tumor Specific Antigens in Serums from Patients with Cervical Carcinoma

Abstract. *Antibody distinct from that involved in neutralization and directed to an antigen (AG-4) induced in HEp-2 cells by infection with herpesvirus type 2 was identified in serums from patients with cervical carcinoma by means of a quantitative micro complement fixation test. The presence of antibody to AG-4 correlates well with the extent of the tumor; antibody is virtually absent in matched control women and in women with therapy and without recurrent neoplasia. Reactivity is not observed with control antigen consisting of a cell extract prepared from uninfected HEp-2 cells. The possible prognostic significance of this antibody and its implications are discussed.*

Herpesvirus type 2 (HSV-2), a virus biologically and antigenically distinct (*1*) from that associated with facial lesions, was isolated from cervical lesions (*2*) and male genitourinary samples (*3*) and was shown to be venereally transmitted (*4*). A relation between HSV-2 and squamous carcinoma of the human cervix has been suggested on the basis of seroepidemiologic studies, indicating a significantly higher prevalence of HSV-2 antibody in patients with both invasive (*5*) and preinvasive (*6, 7*) cervical neoplasia as compared to controls. A similar association was not noted with other venereal diseases (*7*).

In addition to the seroepidemiologic evidence linking HSV-2 and cervical neoplasia, evidence of virus persistence in cervical tumor cells is based on three observations, as follows. (i) Exfoliated tumor cells contain viral antigens (*8, 9*), (ii) a type 2 herpesvirus was isolated from cervical neoplastic cells grown in culture (*10*), and (iii) DNA sequences corresponding to part of the HSV-2 genome were found in a cervical tumor (*11*). Finally Duff and Rapp (*12*) reported the induction of tumors in hamsters inoculated with hamster cells transformed by ultraviolet inactivated HSV-2.

In order to determine whether virus specific products are expressed in cervical tumor cells, we attempted to find, in serums from patients with cervical carcinoma, antibody to HSV-2 antigens other than those involved in neutralization. Such a finding would be analogous to the observation that tumors and cells transformed by SV40 and polyoma virus contain a specific antigen (T) which is distinct from that of the virus particles and which reacts with serums of tumor-bearing hamsters (*13, 14*). As reported here, infection of HEp-2 (human epidermoid carcinoma) cells with HSV-2 for 4 hours leads to the production in these cells of antigen or antigens (AG-4) detected by complement fixation with human serums from patients with cervical neoplasia but not with serums from female control subjects, even though these serums may have equally high levels of antibody to HSV-2, when tested by neutralization.

Serums were obtained from 84 patients diagnosed as atypia (20 patients), carcinoma in situ (19 patients), and invasive cervical carcinoma (45 patients), and from 84 control subjects (from age 20 to 80 years) admitted to the Johns Hopkins Hospital for illness unrelated to cervical cancer and having a negative history of malignancy. These subjects were matched to the carcinoma patients for age, race, sex, and socioeconomic class according to economic deciles of the resident census tracts for the City of Baltimore. Of the invasive cancer patients, 22 women were untreated, and 23 had therapy 2 months to 19 years (mean, 4 years) prior to blood collection. Only one of these (No. 60) had histologic evidence of recurrent neoplastic disease. A third group consisted of women with malignancies at sites other than the cervix. At the time of blood collection, all subjects were free of active genital herpetic infection as determined by Papanicolaou smears and pelvic examination (*15*).

HEp-2 cells were infected with 0.2 to 0.4 plaque-forming units of HSV-2 (*16*) per cell. After adsorption at 37°C, for 1 hour, the cells were overlaid with maintenance medium consisting of medium 199 supplemented with 1 percent fetal calf serum (Grand Island) and reincubated at 37°C for 4 hours. At this time, the cells were collected by scraping, washed with barbital-buffered saline at *p*H 7.4 (*17*), and disrupted by freezing and thawing. Cell debris was removed by centrifugation at 1550*g* for 30 minutes, and the supernatant, designated AG-4, was used in complement fixation tests. A crude extract prepared in the same way from HEp-2 cells treated only with maintenance medium

Table 1. The occurrence of antibody to AG-4, AG-H, HSV-2, and HSV-1 in patients with untreated and treated invasive carcinoma of the cervix.

Group	Tested (No.)	Mean age	Mean economic decile	Positive for anti-AG-4		Positive for anti-AG-H		Positive for anti-HSV-2		Positive for anti-HSV-1	
				No.	%	No.	%	No.	%	No.	%
Invasive cancer	22	52	4.4	20	91	0	0	22	100	18	82
Matched controls	22	50	4.7	2	9	5	23	15	68	19	86
Invasive cancer with											
Radiation	14	53	3.8	0	0	1	7	14	100	13	92
Hysterectomy	9	46	4.7	0	0	0	0	9	100	6	67
Total treated	23	50	4.3	0	0	1	4	23	100	19	82

Table 2. The occurrence of antibody to AG-4, AG-H, and HSV-2 in patients with preinvasive and invasive carcinoma of the cervix and controls.

Group	Tested (No.)	Mean age	Mean economic decile	Positive for anti-AG-4		Positive for anti-AG-H		Positive for anti-HSV-2	
				No.	%	No.	%	No.	%
Atypia	20	34	3.0	7	35	0	0	19	95
Matched controls	20	31	3.2	0	0	0	0	10	50
Carcinoma in situ	19	39	3.4	13	68	0	0	19	100
Matched controls	19	35	3.4	1	5	0	0	10	53
Invasive cancer	22	52	4.4	20	91	0	0	22	100
Matched controls	22	50	4.7	2	10	5	24	15	68
Total cases	61	42	3.6	39	64	0	0	60	98
Total controls	61	39	3.8	3	5	5	8	35	57
Carcinoma of other sites									
Vagina	1			0		0			
Vulva	2			0		0			
Pancreas	1			0		0			
Stomach	1			0		0			
Others	14			0		1			
Total	19			0		1			

and designated AG-H was used as control antigen.

The quantitative micro complement fixation test used was originally described by Wasserman and Levine (*18*); it was adapted to a reaction volume of 0.350 ml, with the use of polyethylene micropipettes and tubes (*19*). Also, $1.5 \times 10^{-4}M$ $CaCl_2$, $1 \times 10^{-3}M$ $MgCl_2$, and gelatin (0.01 percent) were added to the barbital-buffered saline (gel BB++). The assay was performed in duplicate, as described (*19*). Briefly, a mixture of antigen or gel BB++ (for antibody or complement controls), antibody (adjusted so that antibody control gave less than 10 percent complement fixation), and complement (reconstituted lyophilized guinea pig complement, BBL division of Bioquest), was incubated at 4°C for 18 hours, and again at 37°C for 20 minutes. At this time sensitized (*20*) sheep erythrocytes (5×10^7 cell/ml) were added and the mixture was again incubated at 37°C for 60 minutes. After addition of gel BB++ and centrifugation for 20 minutes at 1550*g*, the absorbancy (A) at 416 nm of the supernatant was read. The number of absorbancy units fixed (ΔA) was determined by subtracting the absorbancy of the reaction mixture from that of the antibody control. The percentage of complement fixation was computed by dividing ΔA by the absorbancy of the antibody control. The reaction was considered positive if more than 10 percent of the complement was fixed.

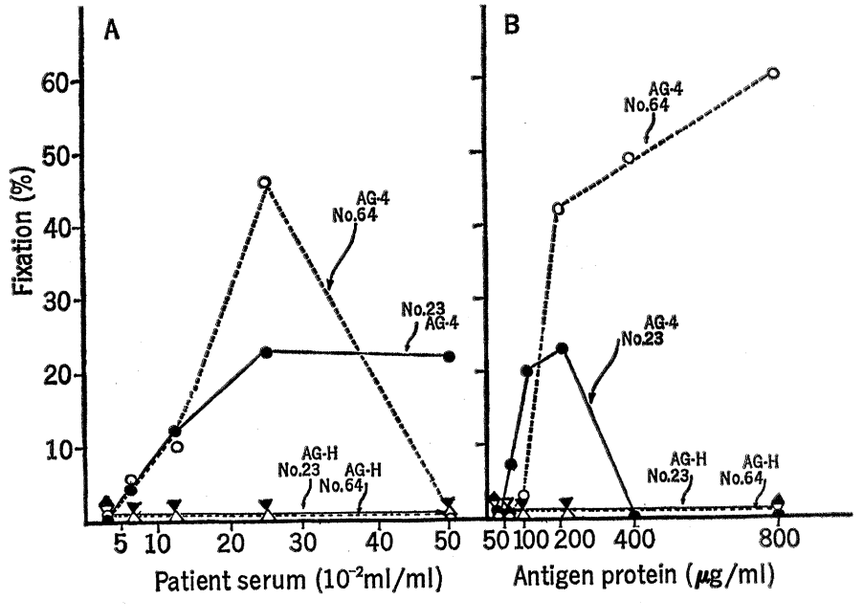

Fig. 1. Micro complement fixation by two serums (*No. 64* and *No. 23*) from patients with invasive carcinoma of the cervix, and antigen AG-4 (○ and ●) or antigen AG-H (△ and ▲). (A) Percentage fixation is plotted as a function of antibody concentration per unit volume of the reaction mixture. Antigen was 20 μg of protein. (B) Percentage fixation plotted as a function of antigen concentration per unit volume of reaction mixture. Subject's serum was 2.5×10^{-2} ml. The diluent was barbital buffer.

Two points emerged from the results of typical complement fixation curves (Fig. 1). First, serums from patients with invasive carcinoma fix complement with AG-4 but not with AG-H. Of the serums tested 87 percent did not fix complement with AG-H; however, to increase specificity further, all serums reacting with both antigens were considered negative for AG-4. Second, maximum fixation usually occurs at a concentration of 200 μg of AG-4 protein as determined by the method of Lowry (*21*). Accordingly, serums were screened against 100 and 200 μg of AG-4 and AG-H proteins, and antibody titer was defined as the reciprocal of the highest dilution of serum fixing at least 10 percent of complement with 200 μg of AG-4 protein.

The occurrence of antibody to AG-4 in patients with invasive carcinoma as compared to a matched control population was determined on a series of coded serum samples, the origin of which was unknown to the person performing the tests. The prevalence of antibody to AG-4 was significantly greater among women with invasive carcinoma of the cervix (91 percent) than among matched controls (chi square: $P < .01$) (Table 1). Of 22 women with neoplasia, 20 had antibody to AG-4, as compared to 2 of the 22 matched controls. On the other hand, as many as 15 (68 percent) of these control serums contained neutralizing HSV-2 antibody. Serums from patients treated prior to blood collection did not have antibody to AG-4. Thus, of 23 women with a history of treated invasive carcinoma, 22, who had radiation or hysterectomy and showed no evidence of recurrent neo-

plasia at the time of blood collection, did not have antibody to AG-4; antibody to HSV-2 was present in 100 percent of treated patients.

The occurrence of antibody to AG-4 in patients with various stages of the disease (Table 2) shows the gradation expected of the development of cervical neoplasia (*22*). Of 20 women with cervical atypia, 7 (35 percent) had antibody to AG-4, as compared to 13 (68 percent) of the 19 women with carcinoma in situ and 20 (91 percent) of the 22 women with invasive carcinoma. On the other hand, antibody to AG-4 was virtually absent in matched controls and in a small number of patients with carcinoma at sites other than the cervix. These included patients with carcinoma of the lung, breast, pancreas, or stomach as well as patients with carcinoma of the vagina, vulva, ovaries, or endometrium, or with adenoepidermoid carcinoma of the cervix. Comparison of the titer of antibody to AG-4 in patients with preinvasive (atypia and carcinoma in situ) and invasive cervical carcinoma indicates that titers cannot be correlated with the stage of the disease (Fig. 2).

Since antibody to AG-4 could not be found in serums from patients treated prior to blood collection, we considered the possibility that antibody to AG-4 might be of prognostic significance. Serums were collected from four patients prior to and at intervals after onset of radiation therapy and were tested for antibody to AG-4 and HSV-2. The results indicated that presence of antibody to AG-4 was associated with tumor recurrence (in two patients); two other patients with no evidence of recurrent neoplasia did not have antibody to AG-4. Therapy did not affect the presence of antibody to HSV-2 (*23*). Unfortunately, serums were not collected at close enough intervals to determine the time that must elapse between therapy and the disappearance of AG-4 antibody.

The focal point of our study is the identification of antibody to HSV-2 induced antigens distinct from neutralizing antibodies in serums from patients with cervical neoplasia and the demonstration that the presence of this antibody correlates with the extent of the tumor. Serums from control subjects and patients with successful therapy were negative. These observations suggest that cervical tumor cells contain antigen or antigens similar or identical to those made early in the productive infection of HEp-2 cells with HSV-2.

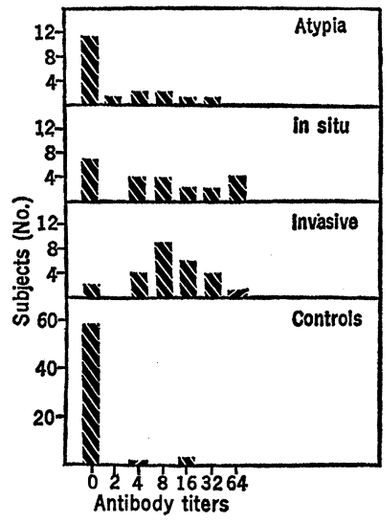

Fig. 2. Distribution of the antibody titers in 122 serums from carcinoma and control groups.

It should be pointed out that AG-4 is made early in infection, is released into the culture fluid 1 hour after synthesis, and does not block the neutralizing ability of antiserums to HSV-2 (*24*). No correlation is observed between antibody to AG-4 and neutralizing antibody to HSV-2 in the serums tested in this study (Table 2).

Although the precise identity of AG-4 must await future studies, the observation that AG-4 does not react with serums from patients with carcinoma of the colon, stomach, or pancreas suggests that it differs from the carcinoembryonic antigen associated with colon cancer (*25*). A nonviron antigen was reported by Tarro and Sabin (*26*) for guinea pig cells infected with HSV-1. Furthermore, Hollinshead and Tarro (*27*) described cross-reactivity between such antigens and an antigen extracted from one case of carcinoma of the lip and one of cervical carcinoma. However the relation between AG-4 and these nonvirion antigens is not clear at present. Thus, unlike the nonvirion antigen, AG-4 is made in HEp-2 cells early in infection and is soluble; and also unlike the nonvirion antigen (*28*), AG-4 is specifically associated with squamous carcinoma of the human cervix (Table 2).

HSV-2 normally kills productively infected cells (*29*). Thus, if a cell is to survive, some of the viral functions must somehow be suppressed. In fact, viral antigens and complete or incomplete virus particles are absent in biopsied (unstressed) cervical tumor cells, and, although exfoliated tumor cells from these patients contain viral antigens, virus particles are not observed (*8*). On the other hand, if HSV-2 has oncogenic potential for human cervical cells, some of its functions must be expressed, particularly those bearing on the transformation of the cells from normal to neoplastic (*14*). A small amount of virus-specific RNA was reported in a cervical tumor (*11*); however, the functions represented are still unknown. If AG-4 is virus coded, the presence of a similar or identical antigen in cervical tumor cells which do not contain structural HSV-2 antigens and virus particles (*8*), would suggest that this represents the rare virus function expressed in these cells. Whatever the ultimate interpretation of the origin of AG-4, the observation (*24*) that serums positive for AG-4 do not react with HEp-2 cells infected with HSV-2 for less than 4 hours, a time at which many of the viral functions are already expressed (*30*), suggests that expression of a rather specific early viral function is associated with cervical carcinoma.

The prognostic significance of the AG-4 antibody must be considered when interpreting these data. Unlike HSV-2 antibody AG-4 antibody was absent in all 22 cases with successful therapy. Furthermore in four cases studied prior to therapy and for 2 years thereafter, presence of AG-4 antibody was always associated with recurrent tumor and in one case with death. These observations, if confirmed in a large number of patients, would suggest that antibody to early HSV-2 antigens may reflect active tumor growth and that separate antibody determinations for "early" and viral antigens might be of diagnostic and possibly prognostic significance.

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Nitrogen Fixation in Termites

Abstract. *Nitrogen fixation, measured by the reduction of acetylene to ethylene, was found in workers of the dry-wood termite* Kalotermes minor. *The soldiers and reproductive castes fixed little or no nitrogen. The fixation rates ranged between 24 and 566 micrograms of nitrogen fixed per month per gram (wet weight) of termite. Nitrogen fixation can be a significant source of nitrogen for these termites.*

Microorganisms living in symbiosis with insects are known to furnish essential vitamins and amino acids required for insect growth and development (*1*). Although their diet can be low in protein, the evidence for nitrogen fixation by herbivorous insects such as termites is not conclusive and has been discounted (*2–4*). The acetylene assay for nitrogen fixation has provided a method for reinvestigating this problem. This assay depends on the ability of nitrogenase to reduce acetylene to ethylene through a reaction which gives a reliable and sensitive measure of nitrogen fixation [see (*5*) for reviews]. Acetylene reduction has been used to detect nitrogen fixation in the guts of ruminants and mammals, including man (*6*). The results indicated that nitrogen fixation is nutritionally insignificant, at least under the conditions of the tests.

Table 1 shows results obtained with *Kalotermes minor*. Since all known nitrogen-fixing organisms are prokaryotic [see (*7*) for a review], it is likely that the nitrogen-fixing agent is the intestinal bacterial flora of the termites. Although the inhibitory effect of anaerobic conditions observed in one experiment (Table 1, assay 4) might suggest aerobic nitrogen-fixing bacteria, the death of the termites in this experiment could also account for the low activities if the bacteria stopped functioning as soon as the termites died. Also notable is the low rate of nitrogen fixation in the soldier and reproductive castes. The different feeding habits of these types might account for the low nitrogen-fixing activity of their intestinal flora. Variations in nitrogenase activity were observed between different batches (Table 1, assays 1 and 2). The cause of these variations remains to be established; the age of the workers might be a contributing factor.

Kalotermes minor, collected from May to October 1972 in San Diego County, California, exhibited a wide range of nitrogen fixation rates under air—from 24 to 566 μg of N fixed per month per gram (wet weight) of termite. Other termites collected during this time, *Cryptotermes brevis* and *Zootermopsis angusticollis*, were able to fix nitrogen; however, only low rates [about 10 μg of N fixed per month per gram (wet weight)] were recorded. If these organisms exhibit variations in nitrogenase activity similar to those observed with *K. minor*, then further collecting might turn up cases of nutritionally significant amounts of nitrogen fixed even in these species. Since fungi play an important role in the nutrition and nitrogen economy of termites (*2*), a symbiotic relation between nitrogen-fixing bacteria and fungi might exist in some termite nests. The cockroach *Periplaneta americana* did not reduce acetylene even following 3 months on a starch or sucrose diet.

It is difficult to ascertain the nitrogen requirements of termites and quantitate the importance of nitrogen fixation. This is due to cannibalism and the fact that the nitrogen content of wood is not directly related to its nutritional value to termites (*2*). From Hungate's data (*2*) on the nitrogen content of termites [an

Table 1. Acetylene reduction by *Kalotermes minor*. The termites were removed on 29 September 1972 from a pine post collected in southeast San Diego the previous day. Live termites were incubated at room temperature (24°C) in stoppered 7-ml serum flasks under the atmospheres indicated below, and the reaction was started by introducing 16.5 percent acetylene. After 150 minutes ethylene was determined by gas chromatography. (The termites in assay 4 died during the experiment.) No ethylene was detected in the absence of acetylene. A factor of 0.25 was used to convert acetylene reduction to nitrogen fixation (*6*). Ethylene determinations were accurate within 3 percent. Acetylene reduction was reproducible for at least three successive days as long as the termites were fed wood; *N*, number of termites per flask.

Assay	Type	Weight (g)	*N*	Atmosphere	Ethylene produced (nmole)	Est. N fixed per month per gram termite (wet weight) (μg)
1	Workers	0.692	66	Air	46	139
2	Workers	1.027	101	Air	108	212
3	Workers	1.155	120	Oxygen (20%)-argon (80%)	141	246
4	Workers	0.819	79	Argon	12	30
5	Reproductives	0.050	5	Air	<0.5	<20
6	Soldiers	0.525	30	Air	2.5	10