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Genetics

Loss of heterozygosity suggests tumor suppressor gene responsible for primary hepatocellular carcinoma

(somatic gene mapping/chromosome 4/recessive oncogenesis)

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ABSTRACT **Primary hepatocellular carcinoma (PHC), epidemiologically associated with chronic hepatitis B virus (HBV) infection, has historically been felt to be caused by the activation or introduction of an oncogene. However, transforming sequences from human PHC have not been reproducibly isolated. In this paper, evidence is presented that suggests PHC may result instead from the loss of an anti-oncogene. Seven of 12 human primary liver tumors tested against a panel of restriction fragment length polymorphisms (RFLPs) demonstrated loss of constitutional heterozygosity for markers on chromosome 4. Tumor and nontumor liver tissue were typed for 11 chromosome 4 RFLPs. In addition, at least one RFLP on nine other chromosomes (1, 2, 6, 7, 9, 11, 13, 14, and 17) was tested for allelic loss. Seven of nine tumors constitutionally heterozygous for chromosome 4q markers showed allele loss in tumor tissue. Six of the seven samples were jointly informative for both 4p and 4q markers. Five of the six demonstrated loss for only 4q RFLPs. In one individual, in which two samples were taken from distant locations within the same tumor, both samples showed loss of the same alleles. Among the other chromosomes informative for allele loss, one tumor showed changes on 13q. No other changes were observed in RFLPs located on the eight other chromosomes tested. These results indicate that an anti-oncogene may be located on 4q and suggest a mechanism for PHC and other cancers seroepidemiologically related to virus infection. Liver cancer caused by chronic HBV infection or other environmental agents may be linked through genetic events responsible for the loss of a tumor suppressor locus (anti-oncogene) located on chromosome 4.**

Primary hepatocellular carcinoma (PHC) is the third most common cause of cancer mortality worldwide, accounting for 250,000–1,000,000 deaths annually (1). Epidemiologic studies have firmly established chronic hepatitis B virus (HBV) infection as an important risk factor for PHC (2–4). Little, however, is known about the oncogenic mechanism. To date, the specific genetic event(s) responsible for transformation in PHC has not been established (5, 6). The role of virally mediated oncogenesis has been extensively explored. As yet, no conclusive results have emerged (see refs. 5 and 6 for reviews). Nonvirally mediated dominant oncogenesis has also been evaluated in PHC. Studies in woodchucks (*Marmota monax*), an animal model for PHC, have shown that three of nine PHC tumors have enhanced expression of a rearranged c-*myc* protooncogene (human chromosome 8) (7). No similar results have been reported in human PHC. Transforming sequences from human PHC have not been reproducibly isolated.

Alternatively, a recessive etiology (8) similar to that observed in a variety of cancers may be involved in PHC oncogenesis. Many solid tumors have been shown to demonstrate genetic changes (loss of constitutional heterozygosity) consistent with a recessive oncogenic mechanism (9–13). A somatic cell hybrid study in an experimental system has provided preliminary evidence that PHC may have a recessive etiology. Hybrids between a cell line derived from a spontaneous mouse hepatoma and normal rat embryo skin fibroblasts do not express a transformed phenotype (14). A suppressing locus (loci) has been localized to rat chromosome 8 based on the evidence that the suppressed hybrid subclones that reexpress a transforming phenotype have lost that chromosome (15). The human analogs for rat chromosome 8 are largely unknown. Preliminary studies in humans provide further evidence for a recessive etiology for PHC. Loss of constitutional heterozygosity in PHC has been reported for loci on chromosomes 4q, 11p, and 13q (16, 17).

MATERIALS AND METHODS

Sample Population. For the purpose of characterizing the genetic constitution of human PHCs, we have tested a collection of probes to anonymous and candidate loci distributed throughout the human genome on a collection of 12 pairs of tumor and nontumor tissue. Samples were obtained from three sources: the Liver Research and Education Foundation (LREF) of the Rancho Los Amigos Hospital, Downey, CA (five tumors); the University of California–Irvine (UCI) (four tumors), and the Liver Cancer Prevention Center (LCPC) of the Fox Chase Cancer Center, Philadelphia (three tumors). The samples were obtained from an ethnically heterogeneous group of patients. The collection includes samples from four South African Blacks (UCI1-4), one American Black (LREF2), three European Caucasians (LREF1, LREF5, LCPC1), one Thai (LREF4), one Tonganese (LREF3), one Japanese (LCPC2), and one Vietnamese (LCPC3). All but one of the tumors in the collection could be shown to be HBV associated. One tumor, for which no serological data were available, did not contain integrated HBV sequences in the tumor DNA (UCI1). All remaining tumors contained integrated HBV sequences.

Restriction Fragment Length Polymorphism (RFLP) Analysis. For each patient, samples of both tumor and nontumor material were obtained. Nontumor material consisted of either uninvolved liver tissue or peripheral lymphocytes. DNA extraction and RFLP screening in both normal and tumor samples were carried out by standard methods (18). DNA was extracted from whole blood and liver tissue by a

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Abbreviations: PHC, primary hepatocellular carcinoma; HBV, hepatitis B virus; RFLP, restriction fragment length polymorphism.

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Table 1. RFLP detecting probes and polymorphic digests used in screening for genetic changes in PHC

Locus	Chromosomal location	Polymorphic digest(s) used	Refs.
REN	1	*Hin*dIII, *Stu* I	22–24
TGFA	2p13	*Rsa* I	25–27
D2S44	2	*Msp* I	28–30
D4S125	4p	*Bcl* I, *Hae* III	28, 31
D4S95	4p	*Taq* I	32, 33
D4S98	4p	*Sac* I	32
D4S10	4p15	*Hin*dIII	34, 35
D4S123	4p	*Hin*dIII	36
KIT	4p	*Hin*dIII	37
D4S67	4cen	*Hin*dIII	38
ALB	4q11–q13	*Hae* III, *Pst* I, *Sac* I	18
IP10	4q21	*Bcl* I	39, 40
EGF	4q25–q27	*Hin*cII	41, 42
FI	4q25–q27	*Bcl* I, *Hin*cII	43
FGB	4q28	*Bcl* I	44, 45
HVBS6	4q32	*Rsa* I, *Taq* I	46, 47
PLG	6q26–q27	*Rsa* I	48
EGFR	7p13–p11	*Hae* III, *Hin*dIII, *Stu* I	49
HRAS	11p15	*Pst* I	50
HBG	11p15	*Hin*cII, *Hin*dIII	51
D13S11	13p11–q11	*Msp* I	52, 53
D13S12	13q21–qter	*Msp* I	52, 54
D14S15	14	*Msp* I	28
D17S4	17	*Pst* I	28, 55

modification of the procedure of Poncz *et al.* (19). After DNA extraction, normal and tumor samples from each individual were digested with restriction enzymes and electrophoresed on 0.8–1.2% agarose gels. The DNA was then transferred to Zetabind filters (AMF Cuno) using 0.4 M NaOH. The filters were prehybridized, hybridized, and washed according to the manufacturer's instructions. DNA probes were labeled by the random primer method of Feinberg and Vogelstein (20) and the blots were autoradiographed using intensifying screens. The probes as well as their chromosomal location and polymorphic restriction digests tested are presented in Table 1 (21–55).

RESULTS

The results of typing probes localized to chromosomes 1 (renin, 2 [D2S44, transforming growth factor α (TGFA)], 4 [D4S125, serum albumin (ALB)], 6 [serum plasminogen (PLG)], 7 [epidermal growth factor receptor (EGF-R)], 11p [β-hemoglobin (HBG), and H-RAS], 13q (D13S11, D13S12), 14 (D14S15), and 17 (D17S4) are summarized in Fig. 1. One of the seven sample sets informative (heterozygous in uninvolved tissue) for the chromosome 13q probes showed loss of an allele in the tumor tissue (LCPC3). Five of five informative samples showed loss of an allele for ALB at 4q11–q12 in tumors. Four of the five samples that showed allele loss for ALB were jointly informative for pYNZ32 on 4p16. None of these tumors showed allele loss for the short arm marker. No tumors showed loss of alleles or aberrant restriction digestion patterns for loci on chromosomes 1 (0 of 8), 2 (0 of 7), 6 (0 of 2), 7 (0 of 4), 11p (0 of 6), 14 (0 of 2), or 17 (0 of 5).

It is possible to determine the significance of the above rates by comparing them with the loss rate observed in other tumors of known recessive etiology. Studies of syntenic RFLPs in the recessive paradigm, retinoblastoma (9, 10), indicate that a loss rate of ≈50% may be expected. It is currently not feasible in PHC to estimate an expected background (noncausal) genome alteration rate related to tumor progression and/or viral disruption. At present, no cytogenetic data are available from nonpassaged PHC tumors. However, cytogenetic studies of a variety of other carcinomas show that individual chromosomes display aberrations in ≈23% of tumors studied (56). Aberrations were defined as change in observed number or structure of a given chromosome and did not reflect changes in ploidy (which would not affect heterozygosity analysis). Therefore, 23% reflects a noncausative (background) loss rate, which may be expected by chance.

Using standard statistical methods, the background and causal change rates can be used to estimate the probability of the observed frequencies of loss and no loss of alleles in tumors. Presented in Fig. 1 are the probabilities of observing the number of allele loss events (and fewer) for each individual chromosome, given a 50% expected allele loss rate. Using an $\alpha = 0.05$ rejection criterion, chromosomes 1q, 2, 11p, and 17 were observed to have significantly less than a

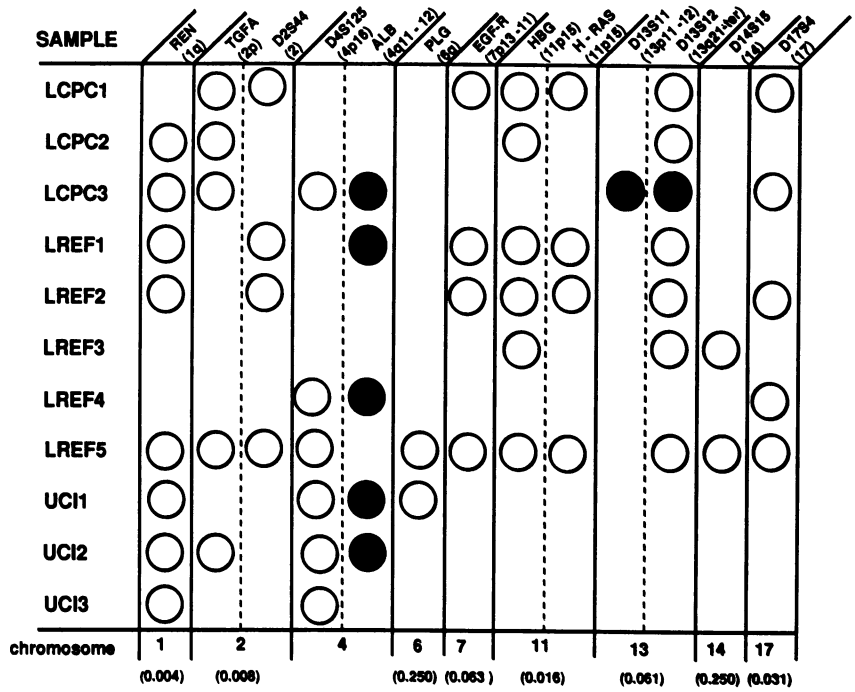

FIG. 1. Loss of heterozygosity results for the chromosome screening panel. ○, The sample was informative (heterozygous in the nontumor tissue) but no allele loss was observed in the tumor sample; ●, loss of alleles in the tumor. Beneath each locus name, in parentheses, is its chromosomal location. Beneath each chromosome, in parentheses, is the probability of observing this many or fewer loss events given an expected rate of 50%.

50% loss rate. However, after adjusting the rejection criterion for the fact that 10 different chromosomes were tested (α = 0.005), only chromosome 1q's observed loss rate was significantly lower than the postulated causal rate. Therefore, chromosome 1q could be judged as uninvolved or excluded from further screening based on statistical criteria. Conversely, the probability of the observed loss rates could be determined assuming the 23% background rate. The 1 of 7 loss observed for chromosome 13q was not significantly different from the background rate (the probability of 1 or loss given a 23% rate = 0.84). The chromosome 4q results, however, were significantly different from the 23% rate ($P < 0.0001$), even after adjustment for multiple tests.

Eleven additional loci mapped to chromosome 4 (refs. 21 and 57; K.H. and J.C., unpublished results) were typed on the sample material in an attempt to increase the number of informative cases and to localize the region of change (see Fig. 2). Two additional tumors were found to demonstrate loss of heterozygosity for chromosome 4 (bringing the total showing loss to 7 of 12). Six of the 7 cases showing chromosome 4 allele loss were jointly informative for one or more markers assigned to the short and long arms. Sample LREF1 showed loss of alleles for both short and long arm markers. Five of the 6 tumors showed loss of alleles only for the 4q loci, thereby localizing the proposed tumor suppressor locus to the long arm. Seven of the 12 cases were informative for a marker (D4S67) localized to the centromeric region of chromosome 4. No loss was observed for any of the informative cases. One sample set, LREF5, had two tumor samples drawn from physically distant localizations. Both samples showed identical loss patterns. An illustration of the chromosome 4 screening is presented in Fig. 3.

Two previous studies have reported loss of heterozygosity in PHC. The first report (16) observed that 3 of 4 PHC tumors showed allele loss at the EGF locus on chromosome 4q. Tests of chromosomal specificity showed that RFLPs on three other chromosomes (nos. 1, 7, and 9) showed no change in tumor tissue. A second study (17) reported that 1 in 5 tumors informative for a marker locus on 4p demonstrated allele loss. In addition, 6 of 14 tumors showed loss of constitutional heterozygosity for chromosomes 11p, 5 of 10 tumors showed allele loss for 13q, 1 in 3 showed allele loss for 10, and 2 of 10 showed allele loss in tumor tissue for chromosome 17. No changes were observed for RFLPs on chromosomes 1q, 2p, 3q, 5, 7q, 9q, 12p, 14q, 15q, 19, 20, 21q, or 22q.

Combining the current study's results with those obtained from the others, it was observed that 6 of 20 tumors showed allele loss for 11p, 6 of 17 showed allele loss for 13q, and 10 of 13 showed loss for 4q. The chromosome 10q, 11p, 13q, and 17q allele loss rates are not significantly different from the background rate. The probability of observing this many allele loss events and more, assuming a random loss frequency of 23%, is 0.54 for 10q, 0.30 for 11p, 0.18 for 13q, and 0.71 for 17q. Conversely, the chromosome 10q, 11p, 13q, and 17q loss rates will be observed with probabilities 0.50, 0.06, 0.17, and 0.05, respectively, when the expected loss frequency is 50%. The chromosome 4q results are significantly different from the background rate ($P < 0.0001$) and suggest a causal rate of 50% or greater.

Pasquinelli *et al.* (46) have cloned a host sequence (HVBS6) adjacent to an HBV integration site mapped by *in situ* hybridization to 4q32. They observed this sequence to be disrupted in 3 of 40 PHCs. The current sample collection was screened with HVBS6 to determine whether alterations at this locus could be involved with the 4q loss of heterozygosity observed in the tumor samples. Uninvolved and PHC tissue sample pairs digested with five different restriction enzymes (*Hin*dIII, *Pst* I, *Rsa* I, *Sac* I, and *Taq* I) were probed with HVBS6. No fragments were observed in tumor tissue that were not present in the uninvolved tissue typed. It is not unlikely, however, that the low frequency of changes previously observed would be missed given the relatively small sample size of the current study (P = 0.39). Two samples (LCP2 and UCI4) constitutionally heterozygous for HVBS6 RFLPs were observed to show allele loss in tumors.

Information on the HBV integration pattern was also available from the tumor samples. A variable number of tandem repeat (VNTR) sequences (28) have been speculated to be preferential HBV integration sites (58). Five VNTR class probes, three of which were derived from HBV sequences (D2S44, D14S15, and D17S4), were used in the screening panel. Two of the VNTRs, D4S125 and D4S95, were located on the short arm of chromosome 4. No evidence of alteration of restriction digest pattern detectable by standard Southern blot analysis was observed in any of the tumors tested. This suggests that neither were large segments

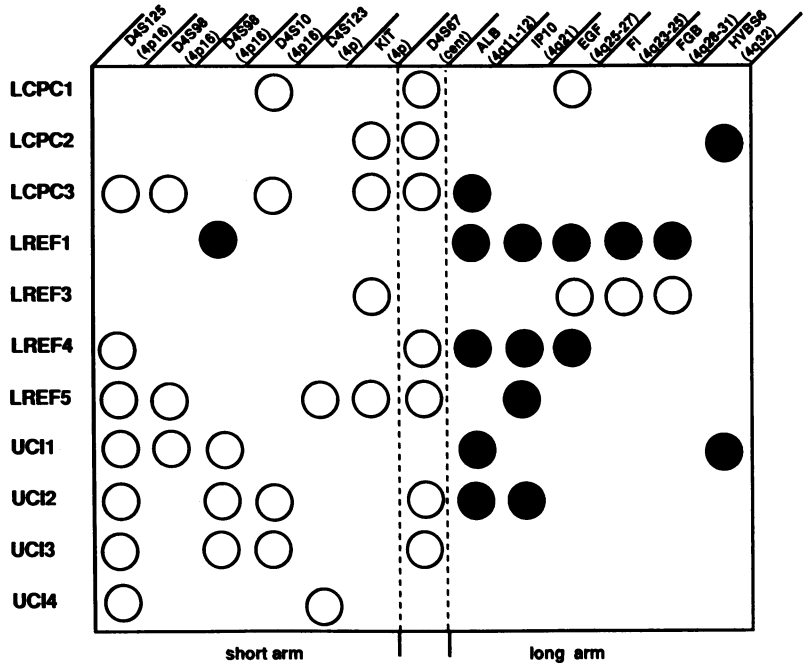

FIG. 2. Loss of heterozygosity results for the panel of RFLP detection probes spanning the length of chromosome 4. ○, The sample was informative (heterozygous in the nontumor tissue) but no loss of heterozygosity was observed in the tumor sample; ●, loss of heterozygosity in the tumor. Beneath each locus name, in parentheses, is its physical location on chromosome 4.

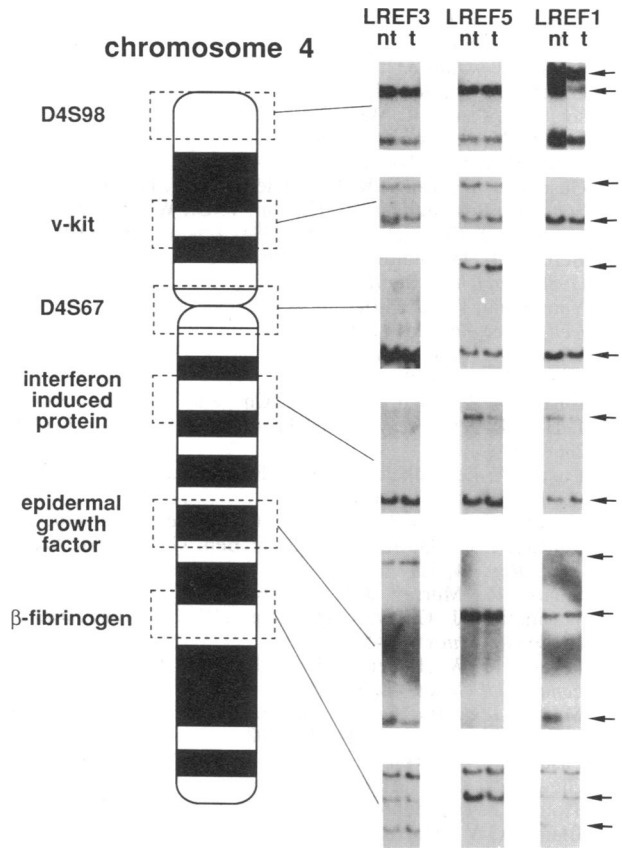

FIG. 3. Southern blot results for three sample sets from six probes spanning the length of chromosome 4. Arrows indicate the polymorphic bands for each RFLP detecting probe. Sample LREF3 is observed to be heterozygous in nontumor tissue (nt) for v-kit, epidermal growth factor, and β-fibrinogen and shows no allele loss in tumor tissue (t) for any of the chromosome 4 RFLPs. LREF5 demonstrates heterozygosity for the markers v-kit, D4S67, and the interferon-induced protein (IP10). Only the q arm marker (IP10) shows allele loss. LREF1 shows constitutional heterozygosity (nt) for D4S98, IP10, epidermal growth factor, and β-fibrinogen. All four probes detect allele loss in tumor tissue (t).

of HBV integrated nor did integration-associated rearrangements occur at these locations in the tumor tissue.

DISCUSSION

The observation that 7 of 12 tumors demonstrate allele loss for chromosome 4 in tumors indicates that a recessive oncogene for PHC may be located on that chromosome. This gene is further localized to the long arm of chromosome 4 by the observation that 5 of 6 tumors jointly informative for allele loss on both arms demonstrate loss only for q arm markers. Two previous studies of allele loss in PHC provide additional support for a 4q tumor suppressor locus. The probability that the 4q results from the combined studies (10 of 13 tumors showing allele loss) would occur given the assumed background rate is <0.0001. The chromosome 4 observations of Wang and Rogler (17) are also consistent with the loss pattern observed in the current sample set. It is expected that only a small portion of samples showing 4q allele loss will also involve changes to the short arm. The short arm loss rates do not significantly differ between the two studies ($P = 0.29$).

The results of the current study do not preclude the involvement of genes located on other chromosomes showing allele loss from playing an important role in PHC. The analysis of the combined results from this and the previous studies suggests that in addition to a tumor suppressor gene on 4q, genes on 10q, 11p, 13q, and 17q cannot be excluded in the etiology of PHC. However, after adjustment for multiple comparisons, with the given pooled sample sizes, six chromosome arms (1q, 2p, 7q, 9q, 14q, 21q, and 22q) can be eliminated as carrying a tumor suppressor locus. Each has a probability of <0.002 of showing a loss rate of 50%.

The recessive etiology for PHC suggested by the observed tumor changes may reconcile experimentally induced PHC observations and epidemiologic studies linking PHC with chronic HBV infection. Model systems of hepatocarcinogenesis have demonstrated that oncogenesis requires both a mitotic activation and a transformation event (59). These steps are evident in two paradigms of human cancer of known molecular etiology, Burkitt lymphoma and retinoblastoma. In Burkitt lymphoma, mitotic activation occurs as a consequence of lymphocyte immortalization by Epstein–Barr virus. A subsequent chromosomal translocation results in transformation. In retinoblastoma, the stage of development provides mitotically active retinoblasts. Subsequent somatic event(s) result in transformation due to loss of functional alleles at the retinoblastoma locus (13q14).

In experimental protocols used for generating hepatocellular carcinoma in laboratory animals (59), the mitotic activation and transformation steps are well characterized. Chemical hepatocarcinogens perform both roles, as they are toxic to the liver and presumably produce DNA mutations. Partial hepatectomy acts to promote mitotic activation. After chemical carcinogenic treatment, nodules of rapidly dividing cells arise. A minority of these nodules persist and eventually give rise to a liver carcinoma.

In human PHC, liver pathology is similar to that observed in experimental systems (for review, see ref. 5). It has been speculated that chronic HBV infection may play a role in human HC similar to that of chemical carcinogens in animal systems (6). HBV may "promote" mitotic activation as a result of hepatocytic necrosis and regeneration due to infection. HBV may also "initiate" DNA alterations as a direct result of viral disruption of the host genome by integration or excision. Chronic HBV infection may also mediate recessive changes through largely indirect processes. The higher hepatocyte turnover rate or functionally larger hepatocyte population resulting from a lifetime of chronic infection may increase the number of opportunities for rare events, such as point mutations and somatic recombination, to disrupt normal gene function.

The results of the current study identify an additional tumor type that may result from loss of a controlling locus rather than activation of a protooncogene. The current results localize this gene to the long arm of chromosome 4. The current study's results, taken in conjunction with those observed by Pasquinelli (46), suggest that this locus may be in the vicinity of 4q32. It is interesting to note that the alteration frequency observed for the HVBS6 locus (3 of 40) is very similar to that observed for the initial probes cloned in the vicinity of the retinoblastoma gene (3 of 37) (60). The observation of a homogeneous genetic change against a background of ethnic heterogeneity of the study population suggests that changes at the 4q locus may represent a common event in PHC oncogenesis. Finally, the establishment of a recessive etiology for HBV-associated PHC could also have important implications for other tumors having seroepidemiologic associations with other virus infections.

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