13. The p19B subclone is a unique 1.0-kb Eco RI intron sequence. Its identity was confirmed by mapping it to the regions Xp22.32-pter and Yq.1-pter by hybridizations to somatic cell hybrid DNA. The restriction map of the cloned sequence p19B and adjacent cloned sequences are consistent with the Taq I and Pvu II RFLP's being due to simple mutations in the enzyme recognition sites.
14. One family included in this study has multiple endocrine neoplasia type 2A. Another family (BD10) segregates Becker muscular dystrophy and the third (DEX1) segregates Duchenne muscular dystrophy.
15. Lod scores [as described by Morton, *Am. J. Hum. Genet.* 7, 277 (1955)] were calculated to determine maximum likelihood estimates for distances between the pseudoautosomal loci and the *TDF*. The confidence intervals were estimated by taking the θ values (recombination fractions) at the maximum lod score ($\hat{z}$) minus 1, as recommended by Conneally *et al.* [*Cytogenet. Cell Genet.* **40**, 356 (1985)].
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Pertussis Toxin Inhibition of B Cell and Macrophage Responses to Bacterial Lipopolysaccharide

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Lipopolysaccharide, a component of the outer membrane of Gram-negative bacteria, activates B lymphocytes and macrophages. Pertussis toxin, which inactivates several members of the G protein family of signaling components, including G_i and transducin, was found to inhibit the lipopolysaccharide-induced responses of the WEHI-231 B lymphoma cell line and the P388D_1 macrophage cell line. These results, combined with the demonstration that lipopolysaccharide inhibits adenylate cyclase activity in P388D_1 cells, strongly argues that lipopolysaccharide activation of cells is mediated by a G_i-like receptor-effector coupling protein.

BACTERIAL LIPOPOLYSACCHARIDE (LPS) is a potent activator of both B lymphocytes and macrophages. Lipopolysaccharide accelerates the phenotypic maturation of pre-B cells and immature B cells to the mature B-cell stage and induces mature B cells to proliferate and differentiate into antibody-secreting plasma cells (*1*). In addition, LPS induces macrophages to secrete immunoregulatory substances including interleukin-1 (IL-1), interferons α and β, and prostaglandins. In conjunction with interferon-γ, LPS induces macrophages to become tumoricidal and bactericidal (*2*). Thus, LPS induces a complicated and extensive set of host immune reactions. Other components of the bacterial cell wall, including peptidoglycan, certain outer-membrane proteins, and exopolysaccharides, have similar properties (*3*). Thus, it is attractive to consider the possibility that higher organisms have evolved mechanisms that enable the immune system to recognize bacterial products, thereby contributing to rapid and vigorous immune responses to bacterial infections.

Little is known about the molecular details by which these bacterial products stimulate B cells and macrophages. We recently showed that LPS can activate the receptor coupling component G_i, which inhibits adenylate cyclase (*4*), in two LPS-responsive murine cell lines: WEHI-231, an immature B-lymphoma cell line, and P388D_1, a macrophage cell line (*5*). LPS inhibited adenylate cyclase in membranes from P388D_1 cells, and, as in other systems in which hormones or other regulatory molecules inhibit adenylate cyclase, this inhibition was abolished by first treating the cells with pertussis toxin, which inactivates G_i by ADP-ribosylating (ADP, adenosine diphosphate) the α subunit of G_i (*4*). Furthermore, it was observed that pretreatment of the membranes from P388D_1 or WEHI-231 cells with LPS and GppNHp, a nonhydrolyzable analog of guanosine triphosphate (GTP), abolished the ADP-ribosylation of G_i by pertussis toxin in vitro (*5*). Because only the inactive form of G_i is a substrate for pertussis toxin, this result suggests that LPS, through its putative receptor, can activate G_i. Because these biochemical experiments indicated that LPS can activate G_i in isolated membranes, we used pertussis toxin to determine whether G_i activation is important for the biological responses of these cells to LPS.

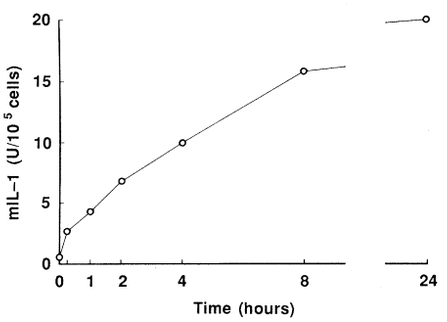

Fig. 1. LPS induction of mIL-1 accumulation in P388D_1 cells. P388D_1 cells (10^6 per milliliter in RPMI 1640 medium containing 10% fetal bovine serum) were cultured with LPS (5 μg/ml) (from *Escherichia coli* 0111:B4; Difco) for the indicated time. The cells were washed twice, fixed with paraformaldehyde (1% in medium for 15 minutes at room temperature), washed twice more, and cultured overnight in medium at 37°C. The IL-1 content of the cells was determined in the thymocyte comitogenesis assay: graded numbers of fixed cells in 0.2 ml were cultured with 10^6 thymocytes from C3H/HeJ mice in the presence of phytohemagglutinin (PHA) (3 μg/ml; Vector Laboratories, Burlingame, CA). After 72 hours of culture, [^{3}H]thymidine (1 μCi per well, 6.7 Ci/mmol; ICN, Irvine, CA) was added, and 4 hours later the cultures were harvested with an automated cell harvester. Incorporation of [^{3}H]thymidine was measured by liquid scintillation counting. One unit of IL-1 was defined as the amount of material required to induce one-third maximal proliferation of the thymocytes stimulated with PHA + IL-1. The error in unit determination was estimated to be less than 25% of the value for each determination reported.

Upon appropriate stimulation, normal macrophages rapidly accumulate a membrane-associated form of IL-1 (mIL-1) before release of soluble IL-1 (*6*). Similarly, the P388D_1 cell line rapidly made mIL-1 upon stimulation with LPS (Fig. 1). Incubation of P388D_1 cells with LPS for as little as 15 minutes induced a measurable increase in the amount of mIL-1 above the basal level found in unstimulated cells. The amount of mIL-1 was increased appreciably within 2 hours of stimulation with LPS and was increased nearly 40-fold after 24 hours.

If the activation of G_i by LPS observed in biochemical experiments reflects a necessary event for the biological response to LPS, then treatment with pertussis toxin should inhibit mIL-1 production. P388D_1 cells were cultured with pertussis toxin for 4 hours and then stimulated with LPS for an additional 2 hours in the presence of the toxin. This toxin treatment totally inhibited the LPS-induced increase in mIL-1 (Fig. 2). In addition, the level of mIL-1 present in unstimulated cells was lowered somewhat. Under these conditions pertussis toxin was a very potent inhibitor of the LPS response; pertussis toxin treatment at only 0.3 ng/ml

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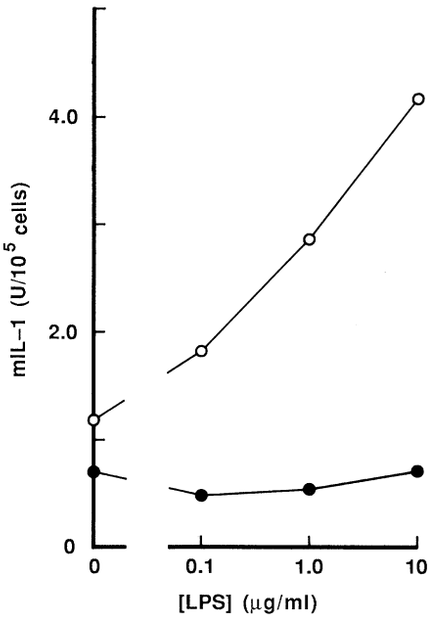

Fig. 2. Pertussis toxin inhibition of LPS-induced mIL-1 accumulation of P388D$_1$ cells. P388D$_1$ cells (10^6 per milliliter) were cultured with (●) or without (○) pertussis toxin (100 ng/ml) (List Biological Laboratories, Campbell, CA) for 4 hours. LPS was added and the cells were incubated for an additional 2 hours. The cells were then fixed and assayed for mIL-1 content as described in Fig. 1.

inhibited mIL-1 induction by 50% (Fig. 3). The completeness of G_i modification by pertussis toxin in the treated cells was monitored by isolating membranes from the same toxin-treated cells and incubating them with ^{32}P-labeled nicotinamide adenine dinucleotide in the presence of additional pertussis toxin. G_i molecules modified in vivo by the toxin would already be ADP-ribosylated and therefore would not be modified with labeled ADP-ribose in vitro. Treatment of P388D$_1$ cells with pertussis toxin at 100 ng/ml for 4 hours caused the modification of more than 99% of the G_i molecules, while treatment with pertussis toxin at only 0.5 ng/ml modified 50% of the G_i molecules (Fig. 3). Thus, the amount of pertussis toxin needed to modify G_i in P388D$_1$ cells closely paralleled the amount needed to inhibit the biological response to LPS. These results support the idea that the action of LPS in P388D$_1$ cells is mediated by G_i.

Because LPS stimulates B cells as well as macrophages, we wanted to find out whether pertussis toxin could inhibit the response of B cells to LPS. For this purpose, we used WEHI-231, an immature B-lymphoma cell line that stops proliferating when its membrane immunoglobulin M (IgM) is cross-linked with antibodies to IgM (anti-IgM) (*7*). LPS protects WEHI-231 cells from this anti-IgM–mediated growth arrest (*8*). These responses may be analogous to tolerance induction of immature B cells induced by antigen or by antibodies directed against immunoglobulin and to LPS-induced maturation of immature B cells, respectively. Pertussis toxin treatment of WEHI-231 cells clearly inhibited the ability of LPS to protect WEHI-231 cells from anti-IgM–mediated growth arrest (Fig. 4). In the experiment shown, toxin-treated WEHI-231 cells responded only as much as untreated cells receiving about 80% less LPS. This was a typical result; in some experiments, however, pertussis toxin completely inhibited the response to LPS. As measurement of this response required incubation of the cells with LPS for a considerable length of time (28 hours), the greater difficulty in inhibiting the response of WEHI-231 cells compared to P388D$_1$ cells may reflect differences in the details of the experiments rather than differences in the inherent ability of pertussis toxin to inactivate the relevant G protein.

The ability of pertussis toxin to inhibit these LPS-induced cellular responses was not simply due to nonspecific toxicity. Thus, pertussis toxin had essentially no effect on either the viability (as measured by trypan blue exclusion) or the proliferation of WEHI-231 cells or P388D$_1$ cells (Table 1). Also, P388D$_1$ cells retained their characteristic morphology after treatment with pertussis toxin.

Thus, in both WEHI-231 B cells and P388D$_1$ macrophage cells, pertussis toxin inhibited the biological responses induced by LPS, an indication that the ability of LPS to activate G_i is crucial for its ability to regulate these cells. However, it has recently become evident that there is a family of G proteins, and pertussis toxin inhibits not only G_i but other members of this family as well. For example, pertussis toxin inhibits transducin, which activates cyclic GMP phosphodiesterase (GMP, guanosine monophosphate) in retinal rods (*9*), coupling components involved in neurotransmitter-induced voltage-dependent ion channels in neural cells (*10*), histamine release by mast cells stimulated with compound 48/80 (*11*), and migration and lysosomal enzyme release in neutrophils (*12*) and macrophages (*13*) stimulated with the chemotactic ligand f-Met-Leu-Phe or with leukotriene B_4. In mast cells, neutrophils, and macrophages, these stimuli appear to act not by regulating adenylate cyclase activity but rather by inducing hydrolysis of phosphatidylinositol-4, 5-bisphosphate (PtdInsP_2) with subsequent mobilization of calcium into the cytoplasm (*11–13*). Thus, there are probably a number of different pertussis toxin–sensitive G_i-like coupling components.

It is not yet clear which of these G_i-like molecules is responsible for the transmembrane signaling generated in response to LPS or, indeed, whether the same component is involved in B cells and in macrophages. However, it has been observed that LPS had no effect on PtdInsP_2 hydrolysis or calcium mobilization in either WEHI-231 cells of P388D$_1$ cells (*14*) or in normal B cells (*15*). This is in contrast to the pertussis toxin–sensitive phosphoinositide responses mentioned above. One model to explain these observations would be that LPS and chemotactic ligands such as f-Met-Leu-Phe

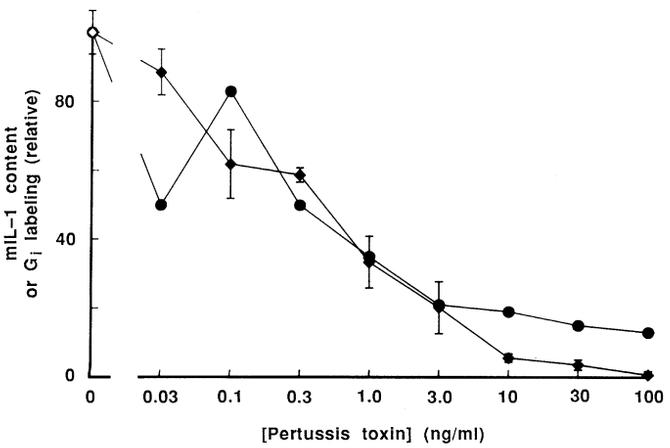

Fig. 3. Comparison of dose response of pertussis toxin for modification of G_i and for inhibition of LPS response of P388D$_1$ cells. P388D$_1$ cells (10^6 per milliliter) were cultured with various concentrations of pertussis toxin for 4 hours. A portion of cells (2×10^6) was removed and stimulated with LPS (5 μg/ml) for 2 hours. These cells were then fixed and assayed for mIL-1 content as described in Fig. 1 (●). The results are expressed relative to the mIL-1 content of cells treated with LPS but not with pertussis toxin (5.2 U per 10^5 cells = 100). Errors in unit determination were estimated to be less than 25% of the value. The remaining cells were used to prepare plasma membranes, which were used as substrate for pertussis toxin–dependent [^{32}P]ADP-ribosylation (*22*). Labeled proteins were separated by SDS–polyacrylamide gel electrophoresis (*23*). The gel was autoradiographed and the amount of ^{32}P incorporated into the 41-kilodalton protein band (G_i) was determined by densitometric analysis of the film (♦). The results shown are the means ± SD of duplicate determinations, normalized relative to the labeling of G_i in membranes obtained from untreated cells (=100).

Table 1. Effect of pertussis toxin on viability and proliferation of WEHI-231 and P388D$_1$ cells. The effect of pertussis toxin on viability was tested by culturing P388D$_1$ cells (10^6 per milliliter) or WEHI-231 cells (10^5 per milliliter) with or without pertussis toxin (100 ng/ml) for 24 hours and determining viability by trypan blue exclusion. The effect on proliferation was tested by culturing P388D$_1$ cells (10^4 in 0.2 ml) with or without pertussis toxin (100 ng/ml) for 12 hours. [^{3}H]Thymidine was added and incorporation was measured 4 hours later as described in Fig. 1. WEHI-231 cells were cultured as in Fig. 4. The results are reported as means ± SD of triplicate cultures.

Cells	Viability (%)		Proliferation ([^{3}H]thymidine incorporation, cpm)	
	Without pertussis toxin	With pertussis toxin	Without pertussis toxin	With pertussis toxin
P388D$_1$	96	95	13,300 ± 2,400	12,500 ± 1,700
WEHI-231	100	100	18,800 ± 200	20,200 ± 2,100
WEHI-231 + anti-IgM (5 μg/ml)	85	82	1,900 ± 700	1,300 ± 400

activate functionally distinct G_i-like components.

It has been reported that anti-IgM induces PtdInsP$_2$ hydrolysis in both normal B cells and in WEHI-231 cells (*15, 16*). Pertussis toxin, however, did not inhibit anti-IgM–mediated growth arrest of WEHI-231 cells (Fig. 4 and Table 1), nor did pertussis toxin, in the amounts we used, have any effect on anti-IgM–induced PtdInsP$_2$ hydrolysis or calcium mobilization in WEHI-231 cells (*14*). Receptor-mediated PtdInsP$_2$ hydrolysis and calcium mobilization in some other cell types is similarly unaffected by pertussis toxin treatment (*17*), in contrast to the situation described above for chemotactic stimuli in neutrophils and macrophages. Thus, it appears that a given second messenger system can be coupled to different transmembrane coupling components in different cell types.

In contrast to our results with WEHI-231, which is an immature B-cell line, Rosoff and Cantley (*18*) reported that LPS induced phosphoinositide hydrolysis and calcium mobilization in the murine pre-B cell line 70Z/3. These biochemical responses were modest, suggesting the possibility that they may be indirect effects of LPS-generated transmembrane signaling events. For example, LPS-activated G_i would inhibit adenylate cyclase, resulting in decreased levels of cyclic adenosine monophosphate (cyclic AMP). In a number of systems, increased cyclic AMP inhibits PtdInsP$_2$ hydrolysis. Therefore, LPS might relieve a preexisting inhibition and thereby cause a modest increase in PtdInsP$_2$ hydrolysis and calcium mobilization. Alternatively, LPS may cause different signaling events in pre-B cells and B cells, perhaps because it binds to different receptors, such as occurs with adrenergic receptors.

Although we have demonstrated that activation of G_i or a G_i-like protein is required for P388D$_1$ macrophage cells and WEHI-231 B cells to respond to LPS, there is no evidence that the critical action of LPS involves regulation of cyclic AMP levels. Inhibition of a signaling system that is normally relatively inactive in unstimulated B cells and macrophages would appear to be an ineffective strategy for activating these cells. G_i could activate other signaling reactions unrelated to cyclic AMP and these could be responsible for generating the biological responses. Even so, the effects of LPS on adenylate cyclase activity may play an important secondary role in the regulation of the cellular responses. High levels of cyclic AMP inhibit many B-cell and macrophage functions (*19*). By inhibiting adenylate cyclase, LPS could allow B cells and macrophages to ignore stimuli that activate adenylate cyclase, such as prostaglandin E_2, which is likely to be found in an inflammatory site. Thus, more work will be required to understand the complex signaling processes induced in these cells by LPS.

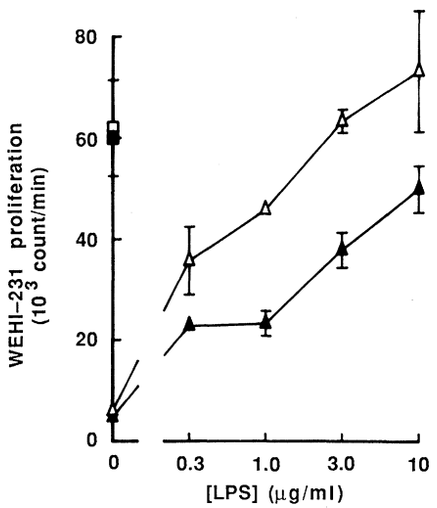

Fig. 4. Pertussis toxin inhibits LPS-induced protection of WEHI-231 cells from anti-IgM–mediated growth arrest. WEHI-231 cells (10^5 per milliliter) were cultured with (■, ▲) or without (□, △) pertussis toxin (100 ng/ml) for 12 hours. The cells were washed once and cultured (10^4 cells in 0.2 ml) with (△, ▲) or without (□, ■) anti-IgM (5 μg/ml) and LPS. Cells treated with pertussis toxin received additional pertussis toxin (100 ng/ml) at the same time as anti-IgM and LPS. The incorporation of [^{3}H]thymidine between 24 and 28 hours after addition of anti-IgM was determined as described in Fig. 1. The results are reported as means ± SD of triplicate cultures.

Pertussis toxin adversely affects many components of the immune system. It was originally observed that pertussis toxin causes an alteration in lymphocyte circulation; this finding led to its being called lymphocytosis-promoting factor (*20*). More recently, as discussed above, pertussis toxin has been observed to inhibit various B-cell, macrophage, neutrophil, and mast cell functions. *Bordetella pertussis*, a Gram-negative bacterium, has components such as LPS that would be expected to activate antimicrobial host defenses through a G_i-like signaling component. By secreting pertussis toxin, *B. pertussis* may be able to disarm the immune system and enhance its chances of survival.

Note added in proof: It has recently been reported (*21*) that pertussis toxin can activate T cells and macrophages. However, these effects required 2 to 10 μg/ml pertussis toxin, which is 20 to 100 times greater than the amount we found necessary to modify 100% of G_i and to inhibit LPS-induced IL-1 accumulation in P388D$_1$ cells. Therefore, it is unlikely that the effects they observed are relevant to the experiments reported here.

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22. Washed cells were disrupted in a Dounce homogenizer (50 strokes) in cold 5 m*M* Hepes (*p*H 7.2), 1 m*M* $MgCl_2$, and 5 m*M* 2-mercaptoethanol. The homogenates were adjusted to 0.2*M* with sucrose and centrifuged at 1300*g* for 3 minutes to remove nuclei. The supernatant was then centrifuged at 230,000*g* for 45 minutes. The pellet (containing plasma membranes) was dispersed in a small tissue homogenizer in 100 m*M* tris (*p*H 7.5). Membrane aliquots (5 μg of protein) were incubated with pertussis toxin [300 ng, preactivated by incubation in 1 m*M* dithiothreitol (DTT) for 30 minutes at 30°C] in 100 m*M* tris (*p*H 7.5), 2.5 m*M* $MgCl_2$, 10 m*M* thymidine, 1 m*M* ATP, 1 m*M* DTT, 1 m*M* EDTA, and 10 μ*M* [^{32}P]NAD (1 Ci/mmol; New England Nuclear) for 60 minutes at 30°C; final volume was 0.1 ml. The reaction was terminated by adding 0.5 ml of cold 90% acetone. The precipitated samples were washed twice with cold 90% acetone, dried, and suspended in sample buffer (*22*).
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Two Avirulent Herpes Simplex Viruses Generate Lethal Recombinants in Vivo

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While it is widely appreciated that infection with a virulent virus can produce disease in an animal, the ability of a mixture of avirulent viruses to produce disease by means of complementation or recombination in vivo has not been established. In this study, two weakly neuroinvasive herpes simplex virus type 1 (HSV-1) strains were simultaneously inoculated onto the footpads of mice. Many (62%) of the animals that received a 1:1 mixture of the viruses died, whereas the animals that received a similar or 100-fold higher dose of each agent alone survived. Of fourteen viruses isolated from the brains of ten mice that died after receiving the mixture of the two weakly neuroinvasive viruses, eleven were recombinants; three of these recombinants were lethal when reapplied to the footpads of mice. These results show that two avirulent HSV-1 variants may interact in vivo to produce virulent recombinants and a lethal infection. They also suggest that different genetic lesions account for the weakly neuroinvasive character of the HSV-1 strains ANG and KOS after footpad inoculation.

MOST STRAINS OF HERPES SIMPLEX virus are neuroinvasive and neurovirulent (*1–3*). When mice are infected on the footpad with such strains, the virus characteristically enters a peripheral nerve and travels centripetally through the nervous system (neuroinvasiveness) where it generally leads to a fatal encephalitis (neurovirulence). However, HSV-1 strains ANG (*4*) and KOS (*5, 6*) are atypical. Although they are fully neurovirulent as measured by intracranial inoculation [median lethal dose (LD_{50}) < 10 plaque-forming units (PFU)], they do not kill mice when inoculated onto mouse footpads, even when susceptibility to the virus is enhanced by treating the inoculation area with hypertonic saline (*7*). These agents may be classified as weakly neuroinvasive, because after rear footpad infection, only small amounts of virus (1 to 10 PFU) may be recovered from the mouse's brain; in contrast, about a million PFU may be recovered from the brain of a mouse infected on the rear footpads with a fully neuroinvasive HSV. The weak neuroinvasiveness of ANG and KOS does not represent a general restriction in mouse cells since both virus strains replicate as well as their virulent counterparts in mouse embryo fibroblasts (at the temperature of a mouse) in vitro and in mouse brains in vivo (*6, 8*).

To determine whether ANG and KOS have different genetic lesions responsible for this behavior in mice and to explore the possibility that two avirulent viruses might interact in vivo to produce disease, these two virus strains were co-inoculated onto murine footpads. If ANG and KOS had different genetic lesions, it was predicted that these viruses might complement each other or recombine in vivo to produce a lethal infection in mice. In two experiments with a total of 26 mice, we found that when ANG and KOS were mixed in a 1:1 ratio and inoculated onto both rear footpads of each mouse (1×10^6 PFU per foot), 62% of the mice died. Yet when the ANG and KOS strains were inoculated alone or on separate feet simultaneously at the same dose, no mice died (Table 1). If mice were given 100 times more than this dose ($\sim 10^8$ PFU) of either ANG or KOS alone, no mice were killed. Therefore, inoculating a mixture of these two avirulent viruses resulted in at least a 100-fold increase in lethality.

We then plaque-purified virus directly from the brains of killed mice to ensure that recombination of the two strains did not occur in vitro. The virulence of these purified viruses was then compared to both ANG and KOS in mice by footpad infection. Four of fourteen viruses isolated from the brains of ten mice produced lethal infections in mice inoculated on the footpad, indicating that recombination may have occurred in vivo in the mice from which the viruses were plaque purified. These viruses were designated AK13, AK23, AK26, and AK27; each was isolated from a different mouse brain (Table 2).

Since ANG and KOS viruses are distinguishable by restriction enzyme analysis of their DNA, the virulent and the avirulent viruses were examined to determine whether they were recombinants. Most of the viruses (11 of 14) isolated from the brains of mice killed by the mixed infection were recombinants. Three of these recombinants, AK13, AK23, and AK27, were virulent. When the DNA's from these three viruses were cleaved with Asp 718 (Kpn I isoschizomer), fragments were detected that comigrated with fragments derived from both ANG and KOS (Fig. 1A). These fragments were assigned to the appropriate parental virus by means of Southern blot analysis (Fig. 1B). Using four different restriction enzymes, we were unable to show that virulent virus AK26, which resembles KOS, was a recombinant since fragments comigrating with ANG-specific fragments were not found (see

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