record (by a factor of 3) is very close to the estimated increase (by a factor of 2.5) determined from a calculation of the export of sulfate from North America eastward, according to emission and deposition modeling (*18*).

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2. We recovered the upper 30 years of core record by using a solar-powered drill. Below this depth, ice core densities were high enough to allow use of a drill powered by a gasoline generator. Generator location with respect to drill site and wind direction was carefully monitored and changed as necessary to prevent local contamination.
3. A network of surface and snow pit studies were used to ensure that Dye 3 contaminants did not affect the drill site (*4*).
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8. Analytical and interpretative details of the data sets not presented in this report, such as concentrations of sodium, ammonium, fluoride, phosphate, and total β-activity, will be presented elsewhere (*4*).
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11. This argument is substantiated by comparison of our core data with that for the University of Copenhagen core 4 km to the northeast. There are differences in the accumulation rate between the two sites only for the period 1966 to 1974, and these are believed to be due to the loss in our core of approximately 2 to 3 years of record within the lower density, more easily damaged section.
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Control of Cachectin (Tumor Necrosis Factor) Synthesis: Mechanisms of Endotoxin Resistance

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Cachectin (tumor necrosis factor) is a macrophage hormone strongly implicated in the pathogenesis of endotoxin-induced shock. The availability of a DNA probe complementary to the cachectin messenger RNA (mRNA), as well as a specific antibody capable of recognizing the cachectin gene product, has made it possible to analyze the regulation of cachectin gene expression under a variety of conditions. Thioglycollate-elicited peritoneal macrophages obtained from mice contain a pool of cachectin mRNA that is not expressed as protein. When the cells are stimulated with endotoxin, a large quantity of additional cachectin mRNA is produced, and immunoreactive cachectin is secreted. Macrophages from mice of the C3H/HeJ strain do not produce cachectin in response to endotoxin. A dual defect appears to prevent cachectin expression. First, a diminished quantity of cachectin mRNA is expressed in response to low concentrations of endotoxin. Second, a post-transcriptional defect prevents the production of cachectin protein. Macrophages from endotoxin-sensitive mice do not produce cachectin if they are first treated with dexamethasone, apparently for similar reasons. These findings give new insight into the nature of the C3H/HeJ mutation and suggest an important mechanism by which glucocorticoids may act to suppress inflammation.

CACHECTIN (*1*) IS A MACROPHAGE hormone produced in large quantities in response to endotoxin (*1–3*) or other stimuli simulating host invasion (*3, 4*). When administered intravenously, cachectin binds to a wide variety of tissues via a specific high-affinity receptor (*1*). After binding, it acts to suppress the expression of several specific messenger RNA (mRNA) species (*5*), thus effecting widespread changes in cellular metabolism. In adipose tissue, cachectin causes complete suppression of the enzyme lipoprotein lipase, thereby preventing the uptake of exogenous triglyceride by fat cells and causing the paradoxical lipemia frequently associated with infection (*6, 7*) or neoplastic disease (*8–10*).

We recently suggested, on the basis of NH_2-terminal sequence data and comparative bioactivity studies, that cachectin and tumor necrosis factor (TNF) are identical proteins (*11*). This identity has been confirmed by genetic sequence analysis (*12–14*) and serves to underscore the broad range of bioactivities attributable to the hormone. We also demonstrated that BALB/c mice passively immunized against cachectin become markedly resistant to endotoxin [lipopolysaccharide (LPS)] (*15*), and have noted (*16*) that animals treated with relatively small doses of cachectin develop metabolic acidosis and a potentially lethal state of shock. These observations suggest that cachectin plays a central role in the pathogenesis of endotoxin-induced shock.

Here we address the mechanism of cachectin induction by endotoxin in peritoneal macrophages. We also analyze the mechanism of endotoxin resistance, both in mice of the C3H/HeJ strain and in macrophages treated with dexamethasone in vitro.

Peritoneal macrophages from normal mice, or from animals primed with thioglycollate 5 days before they are killed, express low but detectable quantities of cachectin mRNA. It is not clear whether this low level of expression is the result of mRNA induction during isolation, or whether the mRNA is actually present in vivo. Immunoreactive cachectin, however, is not detectable in the medium of cultured macrophages prior to induction of the cells by endotoxin. Lysates prepared from noninduced cells also appear to lack the protein in measurable quantities. Once stimulated with endotoxin, peritoneal macrophages express greatly in-

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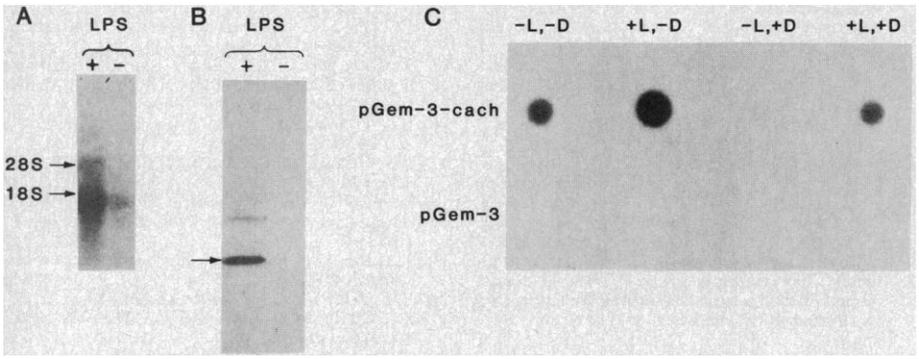

Fig. 1. (A) Detection of cachectin mRNA in BALB/c mouse macrophages by Northern blot analysis (*23*). Arrows point to the 18*S* and 28*S* ribosomal RNA bands. The cachectin probe hybridized to a 17*S* RNA species present in endotoxin-induced cells. The probe also recognized a 28*S* species that has been reported by others (*13*) and may represent a precursor to the 17*S* cachectin mRNA. (B) Modified Western blot analysis of conditioned medium from BALB/c macrophage cultures (16 hours) stimulated with LPS (*23*). The harvested medium was treated as described (*24*). Arrow points to the cachectin band. Above the major reactive species, heavily stained bands represent intermediate products in processing of the prohormone; lightly stained bands [also in (B)] arise through recognition of the primary antibody by the secondary serum. Assay can detect cachectin in concentrations exceeding 20 p*M*, and a linear increase in signal occurs with concentrations between 50 p*M* and 10 n*M*. The reactive band resulted from immunoconcentration of a sample containing approximately 10 n*M* cachectin. (C) Assay of cachectin gene transcription. Macrophages (4×10^8 C3H/HeN; endotoxin-sensitive) were isolated from mice treated 5 days previously with thioglycollate, split into four cultures, and incubated with or without 1 μ*M* dexamethasone (D) for 30 minutes prior to addition of LPS (L) at a concentration of 1 μg/ml, or no LPS as indicated. After 2 hours of further incubation, the cells were lysed in a Dounce homogenizer and the nuclei were isolated by centrifugation at 0°C. Transcription assay was performed as described (*25*). Labeled mRNA was allowed to hybridize for 36 hours at 65°C with a pGEM-3 plasmid containing or lacking (control) the cachectin complementary DNA insert immobilized on nitrocellulose. Film was exposed to the filters for 5 hours at −80°C. Quantitation of binding was performed by Cerenkov counting prior to autoradiography.

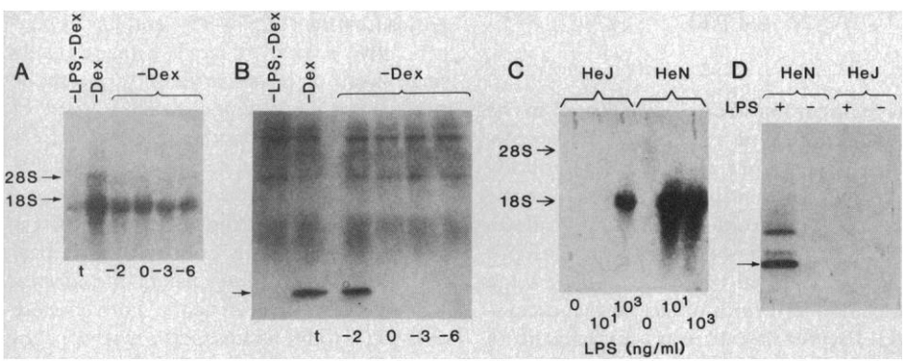

Fig. 2. (A) Northern blot analysis of cachectin mRNA derived from BALB/c macrophages treated with dexamethasone before or after LPS induction. Cells were isolated from thioglycollate-treated BALB/c mice (*24*). After the cells were rinsed with serum-free medium, however, they were treated with dexamethasone (final concentration, 1 μ*M*) 6 or 3 hours before, concurrent with, or 2 hours after induction of the cachectin mRNA by LPS. Controls included a cell monolayer that was not treated with dexamethasone at any time, and a monolayer that was not treated with dexamethasone and not induced by LPS. After induction ($t = 0$), cells were incubated for 8 hours at 37°C in a 5% CO_2 environment. At this time, the medium was aspirated for analysis by immunoblotting [see (B)] and total cellular RNA was extracted, subjected to electrophoresis, blotted, and allowed to hybridize with a nick-translated probe (*24*). Positions of 18*S* and 28*S* ribosomal RNA bands are indicated with arrows. (B) Immunoblot analysis of cachectin production by BALB/c macrophages treated with dexamethasone before or after LPS induction. Medium harvested from macrophage cultures [described in (A)] was subjected to Western blot analysis, with use of the immunoconcentration procedure (*23*). Arrow indicates position of the cachectin band. (C) Measurement of cachectin mRNA synthesis of C3H/HeN (endotoxin-sensitive) and C3H/HeJ (endotoxin-resistant) mouse macrophages. HeN- and HeJ-derived macrophage cultures were prepared from thioglycollate-elicited cells as described in Fig. 1A. Cultures were stimulated with 1000 or 10 ng per milliliter of endotoxin or left unstimulated for a period of 8 hours prior to harvest. Medium was then saved for immunoblot analysis and RNA was extracted, subjected to electrophoresis, blotted, and hybridized with the radiolabeled cachectin probe as described above. (D) Measurement of immunoreactive cachectin secreted by C3H/HeN and C3H/HeJ macrophages. Medium harvested from cultures stimulated with 1000 ng per milliliter of LPS as described in (C) was subjected to the modified immunoblot procedure described in Fig. 1B. Arrow indicates position of the immunoreactive cachectin band.

creased amounts of the cachectin mRNA and secrete large quantities of cachectin into the culture medium (Fig. 1, A and B).

To assess whether the observed increase in cachectin mRNA was attributable to induction of transcription by endotoxin, we performed nuclear transcription assays (Fig. 1C). A threefold increase in the rate of cachectin mRNA transcription was estimated. Thus, transcriptional activation by endotoxin appears to account, in large part, for the elevated levels of cachectin mRNA after induction.

The effects of dexamethasone on the expression of cachectin mRNA and protein were determined. Dexamethasone markedly suppressed the endotoxin-induced increase in cachectin mRNA content, although levels of cachectin mRNA were somewhat elevated compared to levels in noninduced cells (Fig. 2A). A similar depression of cachectin mRNA content was observed whether dexamethasone was added to the cells before LPS induction, at the time of LPS induction, or after LPS induction. Moreover, cachectin gene transcription was strongly suppressed by 1 μ*M* dexamethasone in both induced and noninduced cells (Fig. 1C). Thus, dexamethasone appears to inhibit cachectin gene transcription whether or not the cells have been activated by endotoxin.

Analysis of media from these macrophage cultures revealed a complete absence of immunoreactive cachectin in the medium of cells incubated with dexamethasone prior to or at the time of endotoxin induction, whereas near normal quantities of the hormone were observed in the medium of cells treated with dexamethasone 2 hours after endotoxin stimulation (Fig. 2B). Evidently, dexamethasone acts to inhibit cachectin production at both transcriptional and post-transcriptional levels. However, once the post-transcriptional phase of cachectin biosynthesis has been initiated, dexamethasone is incapable of regulating the process.

We sought to determine whether the transcriptional or post-transcriptional responses to endotoxin were defective in the C3H/HeJ (endotoxin-resistant) mouse. Cachectin mRNA was measured in macrophages from C3H/HeJ and C3H/HeN mice after exposing them to various concentrations of LPS. Macrophages from C3H/HeN mice expressed high levels of cachectin mRNA when exposed to LPS at a concentration of 10 ng/ml. At this concentration, the macrophages from C3H/HeJ mice contained no detectable cachectin mRNA, and nuclear transcription assays confirmed the existence of a defective transcriptional response. However, high levels of cachectin mRNA were expressed by macrophages obtained from both strains when the cells were ex-

posed to LPS at a concentration of 1 μg/ml (Fig. 2C).

In contrast to endotoxin-sensitive macrophages, which are capable of secreting prodigious amounts of immunoreactive cachectin, endotoxin-resistant macrophages produce no detectable cachectin, even when expressing large quantities of cachectin mRNA (Fig. 2D). Lysates of C3H/HeJ macrophages, when subjected to electrophoresis and blotted, also lack the hormone, suggesting that the protein is not sequestered within the cells.

Occasionally, minute amounts of cachectin are detectable in the culture medium of induced C3H/HeJ macrophages after prolonged autoradiography, suggesting that the mRNA can be translated, albeit at extremely low levels. It seems that a mutational event may alter the response of C3H/HeJ macrophages to LPS at two levels. First, higher concentrations of LPS are required in order to elicit high levels of cachectin mRNA within the cell. Second, a post-transcriptional defect prevents the biosynthesis of cachectin even when high levels of cachectin mRNA have been achieved.

Although we have not determined the exact nature of the post-transcriptional control mechanism, control at the level of translation seems likely. We consider that the control of cachectin gene expression by LPS and dexamethasone, and the mutational event preventing cachectin biosynthesis in C3H/HeJ mice, may operate as schematically presented in Fig. 3. Under normal circumstances, the macrophage contains small amounts of cachectin mRNA in a pool which is not translated. The addition of endotoxin mobilizes this sequestered message for translation and stimulates the biosynthesis of additional message. Both of these responses to LPS are defective in macrophages obtained from endotoxin-resistant mice, and in dexamethasone-treated macrophages obtained from animals of normal endotoxin sensitivity.

In C3H/HeJ cells, as in dexamethasone-treated C3H/HeN cells, some expression of cachectin mRNA occurs in the presence of 1 μg/ml of LPS (that is, suppression is incomplete). However, both cell types exhibit a post-transcriptional lesion that is nearly complete. Thus, with regard to their expression of cachectin mRNA and protein, dexamethasone-treated macrophages resemble those obtained from a C3H/HeJ mouse. We are intrigued by the possibility that this resemblance may have a discrete structural basis, in that the protein or pathway that is defective in C3H/HeJ mice might comprise a specific target for the action of dexamethasone in endotoxin-sensitive cells.

The C3H/HeJ mouse does not produce

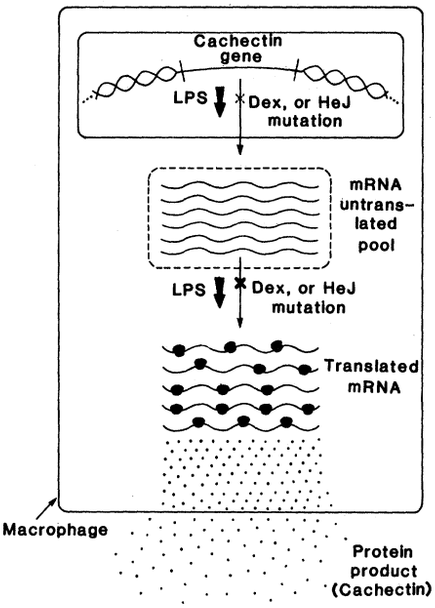

Fig. 3. Schematic summary of the proposed effects of LPS and dexamethasone on expression of the cachectin gene product, and the proposed defect imparted by the C3H/HeJ (*lps*^{d}) mutation. The upper, dashed x signifies a partial transcriptional blockade and the lower, bold x signifies complete blockade.

interleukin-1 (IL-1) (*17*), interferon (*17–19*), or colony-stimulating factor (CSF) (*20*) in response to endotoxin. At present, it is unclear whether the biosynthesis of these other gene products is also arrested at transcriptional and post-transcriptional levels. A single mutational event (the *lps*^{d} mutation) on chromosome 4 has been implicated (*21, 22*) as the basis for the endotoxin resistance of these animals. Possibly, the *lps*^{n} gene product governs the endotoxin-induced production and mobilization of several mRNA species, including those listed above, in response to endotoxin. Storage of these mRNA's in an untranslated pool may serve a protective function, given the toxicity of the protein products involved.

The events that govern the post-transcriptional phase of cachectin biosynthesis remain unclear. Recently, we pointed to the presence of a conserved octameric sequence (TTATTTAT) present in the 3′ untranslated region of mRNA's specifying several endotoxin-inducible inflammatory mediators (*14*), including cachectin (TNF), lymphotoxin, the interferons, IL-1, granulocyte-macrophage–CSF, and fibronectin. We speculate that this sequence may comprise a translational regulatory element involved in the control of cachectin gene expression.

The state of endotoxin resistance conferred by dexamethasone and other glucocorticoid hormones is well known and has had widespread clinical application. However, the efficacy of this approach to the treatment of Gram-negative sepsis in humans has been difficult to demonstrate, and a precise rationale for the use of glucocorticoids has been lacking. In this study we have observed that dexamethasone is only capable of inhibiting the production of cachectin if administered to macrophages prior to endotoxin induction. Once induction has occurred, dexamethasone is incapable of regulating cachectin biosynthesis. As such, glucocorticoids are unlikely to diminish the production of cachectin (thus averting a state of shock) if administered after activation of the host reticuloendothelial system by frank septicemia. Further studies of the regulation of cachectin biosynthesis, at transcriptional, translational, and post-translational levels, may have a major impact upon approaches to the therapy of shock.

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23. A sensitive and specific immunoconcentration assay for cachectin was performed as follows. CNBr-activated sepharose CL4B (Pharmacia) was used to immobilize purified immunoglobulin G from a rabbit immunized against cachectin as described (*15*). An antibody concentration of 5 mg per milliliter of packed beads was achieved. The packed beads (40 μl) were suspended in 2 ml of macrophage medium (prepared as described above) in polypropylene centrifuge tubes. The tubes were shaken gently at 4°C for 4 hours, and after centrifugation (1600*g*) the supernatant was aspirated. The packed beads were boiled in 40 μl of 2× sodium dodecyl sulfate (SDS) gel sample buffer (containing 4% SDS, 0.125*M* tris-Cl buffer, *p*H 6.8, 20% glycerol, and 10% β-mercaptoethanol). The beads, together with the

sample buffer, were then applied to an SDS–polyacrylamide gel (10% to 15% polyacrylamide gradient) and subjected to electrophoresis as described (*1*). Electroblot transfer of the separated proteins onto Schleicher & Schull nitrocellulose paper was carried out with the use of a Bio-Rad apparatus. Blots were immersed in Denhardt's solution [0.3% Ficoll 400, 0.3% radioimmunoassay (RIA)-grade bovine serum albumin (Sigma), and 0.3% polyvinylpyrollidone] for 2 hours and in 1% RIA-grade bovine serum albumin (BSA) (dissolved in Dulbecco's phosphate-buffered saline) for 1 hour. Rabbit antiserum to mouse cachectin was diluted 1:200 in a solution containing 0.85% NaCl, 0.05% Tween 20, 1% RIA-grade BSA, and 0.01*M* tris-Cl buffer, *p*H 8.0 (TTB). The blot was incubated with the antiserum for 3 hours, and then rinsed three times with a solution of 0.05% Triton X-100 and 2% SDS in water. Affinity-purified goat antiserum to rabbit immunoglobulin (Miles-Yeda, Elkhart, IN) was then diluted 1:1000 in TTB, and applied to the blot for 3 hours. The blots were rinsed again with Triton/SDS solution and exposed to ^{125}I-labeled protein A (New England Nuclear) diluted to a concentration of 0.15 μCi/ml in TTB. After 3 hours the blots were rinsed with Triton/SDS solution, then with distilled water, and were dried and used to produce autoradiograms.

24. BALB/c mice were injected intraperitoneally with 3 ml of sterile Brewer's thioglycollate broth (Difco). After 5 days, macrophages were harvested by peritoneal lavage with sterile Hanks balanced salt solution (HBSS), washed once in the same, and plated at a confluent density in 3-cm tissue culture dishes (Becton-Dickinson) with RPMI 1640 medium supplemented with 5% fetal bovine serum (Gibco). After 1 hour, the adherent cells were washed twice with serum-free RPMI 1640 and stimulated by addition of *Escherichia coli* strain 0127:B8 lipopolysaccharide (LPS; Difco) (final concentration 1 μg/ml). Control monolayers did not receive LPS. After 16 hours, the medium was aspirated for use in immunoblotting (Fig. 1B) and the cells were lysed with 5 ml of a solution containing 4*M* guanidinium isothiocyanate, 5 m*M* sodium citrate, *p*H 7.0, 0.1*M* β-mercaptoethanol, and 0.5% Sarcosyl (Pharmacia). CsCl (2 g) was added to each sample, and total cellular RNA was isolated by centrifugation over a 5.7*M* CsCl cushion. RNA (approximately 2 μg per lane) was subjected to electrophoresis in a 1.2% agarose gel containing 2.2*M* formaldehyde as a denaturant. Nitrocellulose blots were allowed to hybridize with a nick-translated pUC-9 plasmid containing a mouse cachectin complementary DNA insert (specific activity = 10^8 dpm/μg). Hybridization was carried out for 12 hours at 43°C in the presence of 50% formamide and 10% dextran sulfate. Blots were then washed with two changes of 2× standard saline citrate (SSC) containing 0.1% SDS, and with two changes of 0.1× SSC containing 0.1% SDS at 60°C, and used in autoradiography.

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Evolution of Human Influenza A Viruses over 50 Years: Rapid, Uniform Rate of Change in NS Gene

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Variation in influenza A viruses was examined by comparison of nucleotide sequences of the NS gene (890 bases) of 15 human viruses isolated over 53 years (1933 to 1985). Changes in the genes accumulate with time, and an evolutionary tree based on the maximum parsimony method can be constructed. The evolutionary rate is approximately 2×10^{-3} substitution per site per year in the NS genes, which is about 10^6 times the evolutionary rate of germline genes in mammals. This uniform and rapid rate of evolution in the NS gene is a good molecular clock and is compatible with the hypothesis that positive selection is operating on the hemagglutinin (or perhaps some other viral genes) to preserve random mutations in the NS gene.

INFLUENZA A VIRUSES HAVE A SINGLE-stranded RNA genome of eight segments of negative polarity, with the shortest segment coding for the nonstructural proteins (NS1 and NS2) (*1*). Figure 1 shows the nucleotide sequences of the NS genes of 15 human influenza A virus strains. The viruses were isolated over a 53-year period and represent all three human hemagglutinin serotypes (H1, H2, and H3). Except for the three Houston isolates, the strains were obtained from diverse geographical locations. The 15 sequences are easily aligned for analysis because of the size conservation of the NS gene segment of 890 bases. Nucleotide substitutions occur at 149 positions scattered throughout the gene and usually, once a base change is observed in a virus isolate, it is found in subsequent strains.

The sequence information as presented in Fig. 1 was analyzed by maximum parsimony (*2*) to determine the phylogenetic tree of minimum length. The best tree found contains a total of 186 substitutions and is illustrated in Fig. 2. The parsimony method also yielded four alternative trees containing 187 substitutions. These alternative trees contain only minor branch perturbations of the best tree.

Figure 3 shows the number of nucleotide substitutions between the origin of the best tree and the tip of each branch (Fig. 2) plotted against the date of isolation of the viruses whose NS gene is represented by that tip. The major line, derived by linear regression analysis, shows that these sequences are evolving at the steady rate of 1.73 ± 0.08 nucleotide substitutions per year, or $1.94 \pm 0.09 \times 10^{-3}$ substitution per nucleotide site per year. The WSN/33 and PR/34 strains appear to have more substitutions per year than expected and therefore were excluded from the evolutionary rate calculation. Since these strains were isolated before refrigeration became available in the laboratory, we believe that continuous passaging in animal hosts and in embryonated eggs (particularly in the first 10 to 15 years after isolation of the strains) may have introduced additional mutations not present in the original isolates. Figure 3 also shows that the group of H1N1 subtype strains, which reemerged in the human population in 1977 and after a 27-year absence (*3*), is evolving at the same rate. These "new" H1N1 viruses have been cocirculating with the H3N2 viruses since 1977 and form a separate evolutionary branch (Fig. 2). In reality, the H1N1 branch should be directly connected to the FW/50 branch of the main tree, since there are only five nucleotide differences between the FW/50 and USSR/77 virus NS genes. However, the viruses were isolated 27 years apart and, on the basis of the calculated evolutionary rate of 1.73 substitutions per year, we would predict approximately 46 additional substitutions in the NS gene of USSR/77 (represented by the broken line in Fig. 2). The observed data thus suggest a unique epidemiology of the new H1N1 isolates.

Several points can be made from the analysis of the data. First, calibration of the molecular clock is not affected by inaccurate paleontological dates, since the time of fossilization (isolation) of these strains is recorded. This may partly explain why the NS gene of influenza A viruses behaves as an accurate molecular clock (*4*). Thus, given only the NS gene sequence of a main line isolate, one can closely estimate the year of its isolation (Fig. 3). Although fewer points are available for measuring the rate in the new H1N1 strains (1977 to 1985), the data (filled squares in Fig. 3) are compatible with a molecular clock ticking at the same evolutionary rate for these NS genes. The mutations seen in the NS genes of the new H1N1 strains (1977 to 1985) are different from those seen in the 1950–1957 H1N1 strains.

The second point that can be made is that

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