A Mouse Macrophage Factor Induces Head Structures and Organizes a Body Axis in *Xenopus*

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Soluble peptide factors have been implicated as the agents responsible for embryonic inductions in vertebrates. Here, a protein (PIF) secreted by a mouse macrophage cell line is shown to change the developmental fate of *Xenopus* embryonic cells. Exposure to PIF causes presumptive ectodermal explants to form anterior neural and mesodermal tissues, including brain and eye, instead of ciliated epidermis. In addition, the induced tissues are organized into a rudimentary embryonic axis. These results suggest that PIF or a closely related molecule is involved in inducing anterior structures and organizing the frog body plan.

EXPERIMENTS BY SPEMANN AND Mangold showed that when a small piece of amphibian tissue from the dorsal blastopore lip is transplanted to the ventral side of a recipient gastrula, it gives rise to another embryonic axis on the belly of the recipient [reviewed in (*1*)]. No other region of the embryo is able to induce and organize a second body axis in this type of transplantation experiment. Spemann named the dorsal lip the organizer principally because (i) it induced the ventral tissues of the host to change their fate and form dorsal mesodermal tissues and a second central nervous system (CNS), and (ii) not only were tissues induced, they were properly organized with clear anterior-posterior (A-P) and dorso-ventral (D-V) polarity. Subsequent experiments have shown that dorsal mesoderm possesses the properties of an organizer by the early blastula stage (*2*). Although the organizer graft is a most dramatic demonstration of inductive interactions, it has since been shown that practically all of the vertebrate body plan arises by a series of consecutive inductions (*3*).

The molecular nature of the signal produced by the organizer is not yet known. Numerous studies over the past several decades have shown that some heterologous inducers (for example, carp swim bladder, guinea pig liver, and bone marrow) have the capacity to induce a second axis when transplanted to a gastrulating amphibian host (*4*). However, the active agent of these various inductors has not been chemically defined (*5*).

More recently, there is increasing evidence that suggests peptide growth factors (PGFs) have an important role in embryonic inductions. When animal pole cells from midblastula *Xenopus* embryos are explanted and cultured in vitro in a balanced salt solution, they differentiate into atypical epidermis (*6*). If the explants are exposed to fibroblast growth factor (FGF) or transforming growth factor–β (TGF-β)–related growth factors, they change their fate and form some mesodermal cell types, notably mesenchyme and muscle (*7*). The normal fate of these animal pole cells is to form epidermis or neuroectodermal derivatives, but not mesodermal tissues (*8*). In the case of XTC-MIF, notochord, segmented muscle, and even some neural tissue is produced (*9*). However, not all mesodermal and neural cell types found along the A-P axis can be induced by these PGFs. For example, none of the PGFs so far tested induce anterior (head) structures such as brain and eye. And, although these in vitro experiments are suggestive, there is no direct evidence that any of these PGFs act as an inducer in the early embryo, and only FGF has been shown to be present in vivo at the time of mesoderm induction (*10*). Moreover, while XTC-MIF can induce a variety of mesodermal tissue types, neither XTC-MIF nor any of the other PGFs has been reported to cause the formation of organized axial mesoderm with clear A-P and D-V polarity.

To find additional molecules involved in pattern formation in early *Xenopus* embryos, we screened 15 cell lines of mouse, human, and frog origin for the ability to secrete substances that induce and organize tissues in animal pole explants. We find that a mouse macrophage cell line, P388D1 (*11*), secretes a strong inducing activity. Other cell lines we tested, with the exception of the F9 teratocarcinoma cell line, which produces ECDGF, did not have any effect on animal pole explants. Explants treated with P388D1-conditioned medium undergo morphogenetic movements similar to those normally seen during gastrulation and form various mesodermal and neural tissues, including brain and eyes. The treated caps have well-organized tissues with A-P polarity. For convenience, we call the inducing activity P388D1-derived inducing factor, or PIF.

Midblastula animal pole explants, consisting of about 100 cells or one-fourth the total blastula cell number, were incubated in cell culture media with or without PIF. Whereas the explants incubated in control medium differentiate only into epidermis, PIF-induced explants frequently look like miniature embryos with a rudimentary axial pattern (Fig. 1). The earliest response to PIF is an elongation that begins 4 to 6 hours after the addition of the factor and mimics gastrulation movements of normal embryos (*12*). During the next few hours explants partially retract, and within 30 hours a cement gland frequently becomes visible at the anterior pole. At this time no other surface structures

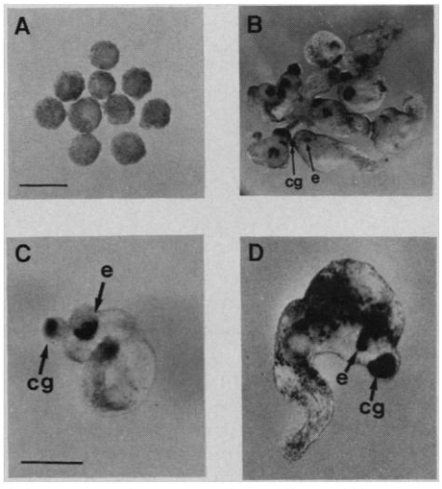

Fig. 1. Differentiation of ectodermal explants from *Xenopus* blastulae cultured in PIF-containing media. Small pieces of presumptive ectoderm (50 to 100 cells) were cut from blastula embryos at stage 7 to 8 (*21*) and incubated in a 1:1 mixture of 0.5× MMR [50 mM NaCl, 1 mM KCl, 0.5 mM $MgSO_4$, 1 mM $CaCl_2$, 2.5 mM Hepes (pH 7.6), and 0.05 mM EDTA (*22*)] with gentamicin (50 μg/ml), and undiluted conditioned media from COS-1 cells (**A**) or from P388 cells (**B**, **C**, and **D**) for 4 hours. Explants were then transferred to 0.5× MMR and allowed to develop for two more days at room temperature. Conditioned media was prepared by culturing cells in serum-free Dulbecco's minimum essential medium (DMEM) (Gibco), containing 2 mM glutamine and gentamicin (50 μg/ml), for 48 to 72 hours. Typical results obtained from at least eight independent experiments are shown. By the end of incubation, some of the PIF-treated explants had differentiated eyes (e), cement glands (cg), and displayed a clear A-P axis with a tail-like structure that twitched from muscular contractions (C and D). These features were never observed in the controls (A). The ectodermal explants and the embryoids they form are much smaller than whole tadpoles. An animal pole explant is only about one-fourth the mass of a intact blastula. The scale bar in (A) also applies to (B) and represent 750 μm; the scale bar in (C) also applies to (D) and represents 400 μm.

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are apparent, but explants twitch, indicating that muscle has formed. One day later, a melanocyte-covered structure resembling a neural tube is often observed. About one-third of the explants develop well-defined pigmented eyes (Fig. 1). In some cases ectodermal folds, normally outlining the tadpole's forehead, are observed. When high concentrations of PIF are used (Fig. 1), induced explants develop predominantly anterior features (head morphology). Highly anteriorized "embryoids" do not have much muscle as judged by histology (Fig. 2). At lower concentrations of PIF, the explants still exhibit an A-P polarity, but the anteriormost tissues (such as eyes) are absent.

Histological sections of PIF-treated explants reveal the existence of distinct differentiated cell types and, in some cases, an organized D-V axis. Notochord is often present through the whole body axis (Fig. 2B). Skeletal muscle is typically found beneath and around the notochord (Fig. 2E). Neural tissue is recognized as darkly stained, compact cells with large nuclei, and in the absence of molecular markers we have identified brain by its characteristic morphology. Brain ventricles (V-shaped cavities) are usually found above notochord (Fig. 2, compare D and F). Sometimes a neural tube continues throughout the whole length of the induced embryoid, and melanocytes (neural crest derivatives) are found nearby (Fig. 2E). The frequency with which these various tissues are observed in PIF-treated explants ($n > 100$) is 82% for skeletal muscle, 70% for notochord, 91% for neural tissue (one-half of which had brain ventricles), and 35% for eyes. In six cases (about 1% of total PIF-treated explants), embryoids developed bilateral symmetry as observed by two closely positioned eyes (*13*).

The induction of eyes is one of the most dramatic effects of PIF and is the strongest evidence that PIF induces anterior structures. None of the other known soluble mesoderm-inducing factors has been reported to cause eye induction in animal cap explants. When animal cap explants are immediately treated with PIF, induced eyes appear about 2 days later; the total developmental time (2.5 days) corresponds to the time of normal tadpole eye development. The percentage of explants that form eyes varies. In some cases 95% of the explants form eyes, but the average over 12 independent experiments is 35%. The reason for this variation is under investigation. Eyes induced in PIF-treated explants have neural retina, pigmented epithelium, and lens and are therefore very similar to normal eyes (compare Fig. 2, C and F). Normally, eye cups form by the sequential inductive influence of pharyngeal endoderm and presumptive heart mesoderm on developing brain (*14*). We find no histological evidence for the formation of pharynx or heart in PIF-treated explants. It is therefore not easy to reconcile the current view on eye induction with our observation that, in some cases, an eye is formed in induced explants in the apparent absence of any other visible structures except mesenchyme and epidermis (Fig. 2C).

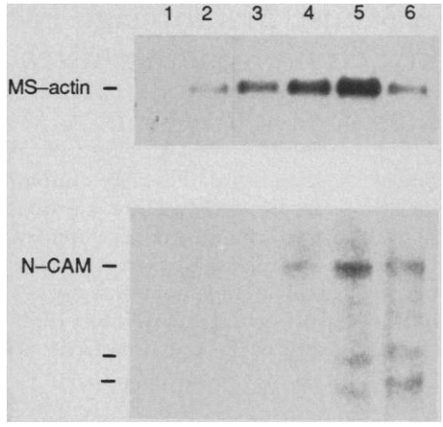

Fig. 3. Northern blot analysis of RNA extracted from ectodermal explants induced by PIF. Explants were incubated in 0.5× MMR (lane 1) or in increasing concentrations of partially purified PIF at 1:1600, 1:180, 1:60, and 1:20 dilutions corresponding to lanes 2, 3, 4, and 5, respectively. Lane 6 is a control containing tadpole RNA. Total RNA isolated (five explants worth per lane) was run in a formaldehyde-agarose gel, transferred to GeneScreen, and hybridized to ^{32}P-labeled antisense RNAs to muscle actin [muscle-specific (MS)] and N-CAM (neural-specific) genes as described (*24*). There is only a single muscle actin–specific transcript and three N-CAM–specific transcripts (*16*).

To confirm and extend the histological assays, we performed RNA blots to test for the presence of muscle-specific actin mRNA (*15*) and neural-specific neural cell adhesion molecule N-CAM mRNA (*16*). A dose-response curve of RNA isolated from PIF-treated animal caps shows that the amount of muscle actin mRNA increases with increasing PIF concentration. N-CAM transcripts appear only at high concentrations of PIF (Fig. 3). These results suggest that low concentrations of PIF can induce mesoderm tissue (muscle), but not neural tissue.

An initial characterization of PIF was conducted to determine its biochemical properties. Animal pole explants (eight per group) were incubated in P388D1 cell-conditioned medium or with medium that had been treated for 1 hour at 37°C with trypsin (200 μg/ml). Ninety percent of PIF activity is lost on incubation with trypsin, but not after incubation with both trypsin and soybean trypsin inhibitor (0.4 mg/ml). Trypsin inhibitor alone does not show any inducing activity. PIF is also heat stable: crude conditioned medium can be boiled for

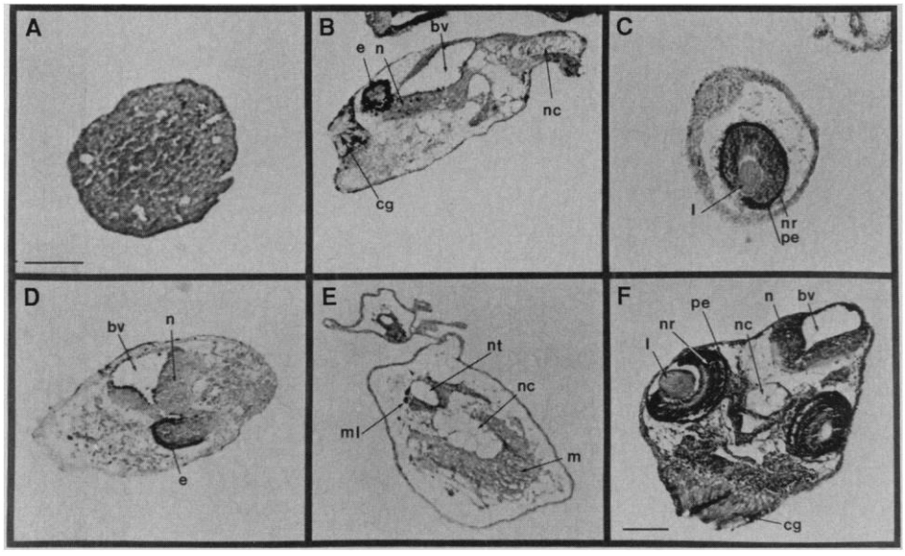

Fig. 2. Histological sections of ectodermal explants allowed to develop for 2 days. (**A**) Control explant incubated in 0.5× MMR salts only. (**B**, **C**, **D**, and **E**) Explants treated with PIF. (**F**) A transverse section through the head of normal tadpole. Samples were fixed in Bouin's solution, embedded in Paraplast, sectioned (7 μm), and stained with hematoxylin-eosin as described (*23*). Brain is evident in PIF-treated explants (B and D, compare to F) where compact neural tissue forms ventricles. Optic cups are evident (B, C, and D) with heavily pigmented epithelium surrounding neural retina. In some cases the neural tissue forms a tube (E) going throughout the explant, whereas in other instances it remains a large mass of tissue (D). Abbreviations are as follows: bv, brain ventricle; nc, notochord; n, neural tissue; m, muscle; l, lens; nr, neural retina; pe, pigmented epithelium; and ml, melanocytes. The scale bar in (A) applies also to (C), (D), and (E) and represents 150 μM; the scale bar in (F) also applies to (B) and represents 100 μM.

15 min without any detectable loss of inducing activity.

To purify PIF we used hydroxylapatite, phenyl-Sepharose, and DEAE-Sepharose columns and achieved a 20-fold purification (Table 1). Nevertheless, gel electrophoresis of the active fractions from DEAE-Sepharose show the presence of only a few proteins (Fig. 4C, lane 1). When the active fractions from DEAE-Sepharose are applied to a Superose-12 fast protein liquid chromatography (FPLC) column, PIF elutes as a single peak and migrates with 25- to 30-kD proteins (Fig. 4A). In separate experiments, when the active fractions from DEAE-Sepharose are fractionated by reversed-phase high-performance liquid chromatography (HPLC), PIF is separated from a major protein contaminant (Fig. 4B). Gel electrophoresis of active fractions from the reversed-phase HPLC reveals only one major band that is the correct size for PIF (~28 kD, Fig. 4C, lane 2). Also, when a nonreducing gel is loaded with the active fraction from DEAE-Sepharose and the fractionated proteins are eluted from gel slices, PIF activity is only found in the slice containing this 28-kD band (Fig. 4C). Finally, gel electrophoresis with dithiothreitol (DTT) shows that the putative band of PIF is reduced to bands of about 15 kD. This suggests that PIF may be a dimeric protein held together by disulfide bonds. Complete purification and sequencing will be required to fully characterize PIF.

P388D1 cells secrete PIF into the culture medium constitutively. PIF is recovered from cells grown in the complete absence of serum and without stimulation by bacterial lipopolysaccharide. P388D1-conditioned medium has a titer of up to 1:100, that is, the inducing activity is still evident at a 1:100 dilution, as measured by early elongation of treated explants. P388D1 cells are known to secrete a variety of different lymphokines (*17*); some of them [interleukin-1 (IL-1), IL-6, and granulocyte-macrophage colony-stimulating factor (GM-CSF)] were tested and found to be inactive in our induction assay.

PIF is biochemically distinct from other known peptide mesoderm-inducing factors. Unlike FGF-related mesoderm-inducing factors (7), PIF does not bind heparin. PIF containing medium was passed through a heparin-agarose column equilibrated with 20 mM tris-HCl, pH 7.6, and 50 mM NaCl. PIF did not bind to the column, whereas FGF (50 ng/ml) added to control medium is entirely retained on the column. Although we have not compared the chromatographic properties of XTC-MIF and PIF side by side, we have found that XTC-MIF elutes from DEAE-Sepharose at 0.4 M NaCl (*18*), whereas PIF elutes at 0.2 M NaCl (Table 1). Secondly, XTC-MIF is eluted from phenyl-Sepharose by 30% ethylene glycol (*18*), whereas PIF remains bound and elutes only at 50% ethylene glycol (Table 1).

PIF is also different from other mesoderm-inducing factors in terms of its inducing properties. FGF does not induce notochord, and neither TGF-β2 nor XTC-MIF have been shown to induce eyes or other anterior neuroectodermal derivatives. PIF is unusual in that it can induce an organized axis with A-P and D-V polarity. In all, these data suggest that PIF is a novel inducing factor. However, until PIF is purified and sequenced we cannot rule out the possibility that PIF is one of the already known PGFs. We have found (*19*) that another TGF-β–related factor, activin, has a strong mesoderm-inducing activity, but it is not yet known if PIF and activin are identical (*19*).

It is unclear whether PIF by itself can induce neural tissues without first inducing mesodermal tissues. In some cases (Fig. 2C), we find neural derivatives (eye) without any obvious mesodermal tissue. In the absence of molecular or unequivocal histological markers for head mesoderm or pharyngeal endoderm, we cannot rule out the presence of those tissues in induced animal caps. And the conventional view, based on a large body of embryological evidence, is that all neural tissue is induced by mesoderm. However, Sato and Sargent (*20*) suggest that a neural marker, N-CAM, can be in-

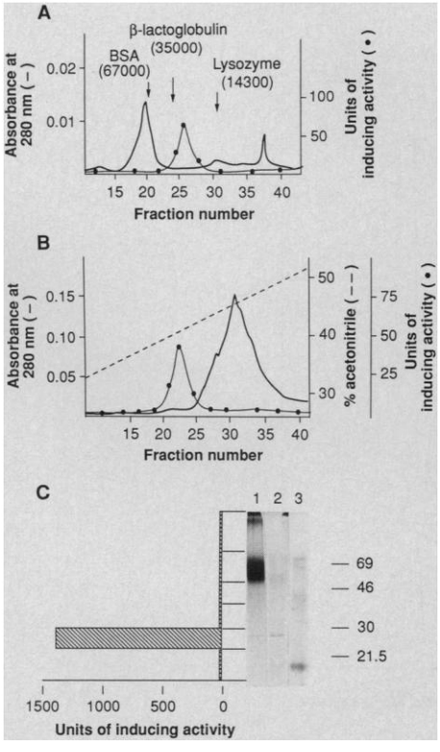

Fig. 4. PIF corresponds to a 28-kD protein. (**A**) PIF-containing fractions eluted from DEAE-Sepharose (see legend to Table 1) were concentrated in Centriprep 30 (Amicon) and applied to a Superose-12 FPLC column equilibrated in 0.1 NaCl and 20 mM tris-HCl, pH 8.0. BSA, bovine serum albumin. (**B**) PIF-containing fractions eluted from DEAE-Sepharose were applied to a Vydac C4 reversed-phase HPLC column, equilibrated with 0.1% trifluoroacetic acid (TFA). Elution was performed with a gradient of 0 to 95% acetonitrile in 0.1% TFA and PIF activity eluted at about 40% acetonitrile. (**C**) Active fractions were analyzed by nonreducing SDS–polyacrylamide gel electrophoresis in an 11% acrylamide gel: DEAE-Sepharose eluate (lane 1) and reversed-phase HPLC eluate (lane 2). Lane 3 is identical to lane 2 except that DTT was added to the loading buffer. Gels were stained with silver nitrate (Bio-Rad). Molecular size markers are shown on the right. To find out which protein band corresponds to PIF, we sliced a gel identical to lane 1 into six pieces, and each slice was incubated overnight in 20 mM tris-HCl (pH 8.0) and 8 M urea. After concentration by Centricon-30 and precipitation with four volumes of acetone, protein was dissolved in 8 M urea, diluted with 0.5× MMR and assayed for PIF activity. Results of the induction assay are shown in the bar graph to the left of the gels.

Table 1. Partial purification of PIF. Culture medium conditioned by P388D1 cells for 72 hours in the absence of serum was concentrated 50-fold on Amicon RA-2000 concentrator (10,000 molecular weight cut off). After heating in a boiling water bath for 15 min, the solution was cleared by centrifugation at 11,000*g* for 20 min and loaded onto hydroxylapatite column (Ultragel, Bio-Rad) equilibrated with 0.05 M potassium-phosphate buffer, pH 7.0. A gradient of 0.05 to 0.6 M potassium-phosphate, pH 7.0, was used for elution, and PIF came off at 0.2 M potassium-phosphate. Active fractions were applied to phenyl-Sepharose column (Pharmacia), equilibrated with 0.1 M potassium-phosphate, pH 7.0, and eluted with a gradient of 0 to 8 M urea in 20 mM tris-HCl, pH 8.0. Activity eluted at 6 to 8 M urea. In other experiments, PIF was eluted from phenyl-Sepharose by 50% ethylene glycol. Active fractions from phenyl-Sepharose were loaded on DEAE-Sepharose column equilibrated with 0.05 M NaCl and 20 mM tris-HCl, pH 8.0, and eluted with a gradient of 0.1 to 0.6 M NaCl. Inducing activity was found in 0.2 M NaCl fractions. Protein was determined as described (*18*).

Purification step	Total PIF activity (U*)	Total protein (mg)	Specific activity (U/mg)
Crude conditioned medium	160,000	1,440	111
Hydroxylapatite column eluate	80,000	380	210
Phenyl-Sepharose column eluate	16,000	12.4	1,290
DEAE-Sepharose column eluate	6,600	3.2	2,060

*One unit of PIF is defined as the minimal amount present in 1 ml of medium that will induce all of the animal caps. Thus, 100 U of PIF can be diluted 1:100, and the diluted material will still induce mesoderm in explants.

duced in dissociated and reaggregated embryonic cells without apparent mesoderm induction (as judged by the lack of muscle-specific actin expression). This result raises the possibility that neural tissue may be induced directly under in vitro conditions.

Our data show that PIF induces differentiated cell types in animal pole explants and that these tissues show an axial polarity. An important question is, how does the observed polarity in PIF-induced embryoids arise? At least two possibilities exist. Either PIF itself has polarizing activity, and cells respond to it according to a concentration gradient. Alternatively, the ability of a cell to respond to PIF may be predetermined, perhaps by the position of the cell in normal embryos. Lineage tracing of induced explants, as well as dissociation and reaggregation experiments, may help clarify this issue.

Our data suggest that the basic body plan of the embryo can be formed as a result of the action of a single factor. Moreover, when P388D1 cells are implanted into the blastocoel of a gastrula, the host forms a second dorsal axis, as it does after implantation of an organizer (*1, 13*). Thus, one signal appears to be sufficient to trigger axial organization and patterning of epidermal, mesodermal, and neural tissues. This is consistent with Neuwkoop's proposal that a graded distribution of a single inducing factor causes tissue patterning (*6*). Whether the administration of PIF or a related factor results in the subsequent synthesis and release of other factors necessary for full axis formation remains to be determined.

Finally, the effects of a mouse-derived factor, PIF, on frog development raises the question of whether PIF is conserved across species. It will therefore be interesting to learn whether PIF functions in early mouse development. At the same time, identification of a PIF homolog in Xenopus will allow us to determine when and where this protein is normally expressed. That information will give the first indication of whether PIF acts as mesoderm or neural inducer, or both, and what, if any, relationship PIF has to the Spemann organizer.

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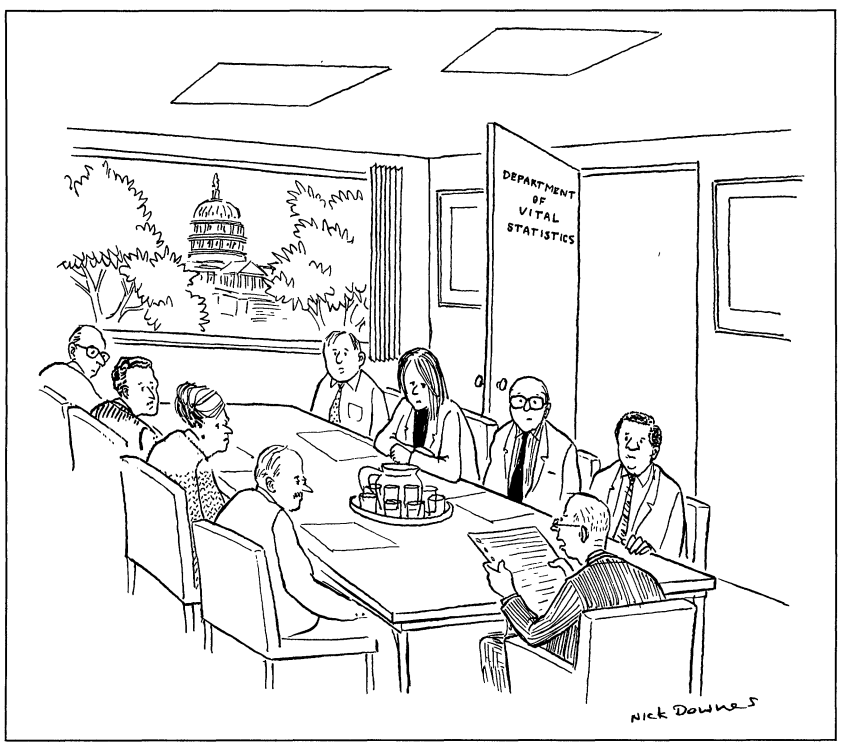

"It seems that only one of you is giving 100%, two are giving 80% and the rest are giving 69% or less."