

Embryogenesis of chimeras, twins and anterior midline asymmetries

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Human spontaneous chimerism, with one body built from cells of both twins of a dizygotic (DZ) pair, is supposed to be extremely rare, arising from the exchange of blood cells through placental anastomoses. Mosaicism is supposed to be far more common, arising from single zygotes by embryonic mutation. Because typical diagnosis of mosaicism can neither identify nor exclude chimerism, 'mosaicism' may often be chimerism undiscovered. Evidence shows chimerism arises primarily from DZ embryo fusion and is not rare, although it has negligible probability under the hypothesis of independent double ovulation and independent embryogenesis. If, instead, DZ twin embryos begin development as a single cell mass, chimerism is likely. This would be consistent with observations that DZ twins develop as differently from singletons as monozygotic twins do with regard to embryogenic establishment of asymmetries of midline neural-crest-driven structures of brain, face and heart. Chimerism is a significant component of human embryonic development that deserves closer attention as a mechanism of developmental variation. The 'common knowledge' understanding of twinning mechanisms is at best inadequate. The importance of the difference lies in what we can learn from chimerism about human embryogenesis and the cellular origins of structures and functions basic to the business of becoming human.

Key words: twins/chimeras/mosaicism/anterior midline asymmetries/human embryogenesis

Introduction

Spontaneous human chimerism has lately drawn increasing notice. As a plot device in television crime drama, the victim knows exactly who hurt her, but DNA from his cheek swab indicates that he is only a brother to the source of the DNA from the rape kit ... until his chimerism is discovered. A world-class athlete accused of boosting his endurance by transfusion of extra red blood cells tried to excuse the extra antigens in his samples as a spontaneous sole-survivor twin chimerism (Henderson, 2005). His defence was dismissed, perhaps for the wrong reasons. A woman needing an organ donor was told that two of her three sons were appropriately related to each other, but not to her ... until she was found to have a germ-line chimerism producing two different families of germ cells (Yu *et al.*, 2002). When boy–girl twins (opposite sex, OS = proof of dizygosity) are delivered in a single chorion (monochorionicity, MC = proof of monozygosity) (Miura and Niikawa, 2005), astonished questions arise—the only credible answer to which seems to be that the cells from which those dizygotic (DZ) twins developed were together in a single mass of cells around which a single trophoblast/chorion differentiated during the first few days of embryogenesis. They grew from there into separate bodies, with one or both of them carrying souvenir

cells of the other's genotype. Their reciprocal chimerism is discovered only because of investigations of that MC-OS-DZ discrepancy.

Chimeras are not visibly different from the rest of us unless a developmental anomaly in one of the cell lines, or sex discordance between the cell lines, sometimes causes a visibly abnormal phenotype. Without such cause for notice (as would usually be the case), they are impossible to differentiate from single-genotype people by ordinary observation and seriously difficult to identify even with the best of the newest biomedical technologies. Cases are discovered in the population with low frequency and high technical difficulty, creating the pervasive false impression that they are rare. Critical consideration of their cellular origins should improve understanding of human developmental biology, especially with respect to the cellular origins and developmental consequences of twinning, and the intimately related establishment of normal asymmetries of structure and function. Much of what is offered as biological background is not supported by physical evidence and is probably wrong. The object of this work is to assemble the available evidence into a coherent and useful idea of what we should learn from the special embryogenic events that lead to the development of these special people.

Subjects of the analysis

We are concerned here only with spontaneous chimerism in individuals whose mixed cell lines arose without medical artifice—not from transfusion or other tissue transplantation. Because experimental chimeric mammals and birds have been powerful tools for studies of developmental biology, some of their characteristics will be mentioned to help with understanding what we might expect to see in, and learn from, spontaneous human chimeras.

Churchill's Medical Dictionary (1989) defines a chimera as: 'an organism composed of two or more genetically distinct cell types.' In her review of the biology of the human chimeras known in 1983, Tippet (1983) says: 'a chimera has cells from two or more zygotes.' The definition in Churchill's Medical Dictionary (1989) mentions somatic mutation as a possible source of chimerism, but goes on to say: 'it occurs in humans most commonly when the blood of dizygous twins mixes *in utero*.' The definition in the Online Medical Dictionary (2004) does not mention mixing of bloods, but offers fusion of embryos first among the possible origins suggested.

Chimeras = Mosaics? Mosaics = Chimeras? Both? Neither?

In the Online Medical Dictionary (2004), 'chimera' is the last word in their definition of 'mosaic'. In Anderson *et al.* (1951), we find: 'a mosaic is formed of cells of a single zygote lineage.' Churchill's Medical Dictionary (1989) defines 'mosaic' as: 'in genetics, an individual whose cells consist of at least two genotypically distinct populations that arose after fertilization through somatic mutation or somatic nondisjunction.'

In the actual everyday practice of clinical genetics, a diagnosis of 'mosaicism' results from cytogenetic analysis carried out for prenatal diagnosis or for explaining a congenital phenotype which a clinical geneticist believes might represent an aneuploidy. Bodies composed partly of normal cells and partly of chromosomally abnormal cells are not very rare in such situations, appearing with a frequency in direct proportion with the clinical intuition of the geneticist choosing patients to be tested in that way. No such investigation is made with regard to 'multifactorial' or single gene anomalies. In neither case can I find any consideration that phenotypic variation might ever be due to mixed genotypes and proportional to the fractions of abnormal versus normal cells.

Therefore, 'mosaicism' is—not by theoretical definition, but as a matter of everyday clinical genetic understanding and practice—a cytogenetic phenomenon.

When a newborn, or an adult never properly diagnosed before being found in an institutional population, shows signs of chromosomal anomaly, blood samples are taken in the expectation of finding an abnormal genotype to explain the phenotype. Sometimes many, even most, of the cells are normal and the diagnosis is 'mosaicism'. If the technicians cannot find at least two or three identically abnormal chromosome sets in cells from 50 white cell clones, then the patient will often lose a pinch of skin from under each arm to provide fibroblasts for culture and further testing. Some mosaicisms not detectable in blood do show up in skin, often with different normal versus abnormal proportions in samples from the two arms. When no

evidence of the expected anomaly can be found in the blood or skin of such a patient, the belief usually lingers that there are abnormal cells in there somewhere—either in tissues not sampled, or previously active in embryogenesis but having died off to a presently undetectable level. We do frequently find cell line fractions in samples from 'mosaic' individuals varying over time (Hansen *et al.*, 1984) and we have, after all, examined only a few cells from only one or two tissues.

When the technicians find the all-aneuploid or part-normal-part-aneuploid mixture of cells that they sought, the search is over. Samples are usually not tested for differences other than those found in the karyotype. The studies that typically yield a diagnosis of mosaicism do not expect chimerism, can seldom recognize it, and cannot exclude it. The laboratory may be motivated to additional efforts by certain sex chromosome differences between the cell lines, or the obvious involvement of more than one chromosome, such that a single segregation anomaly becomes an implausible answer (Wiley *et al.*, 2002).

The cell line differences typically observed in mosaicism are supposed to have arisen from post-zygotic (mitotic) error. Some change is supposed to have occurred in one of the cell divisions in embryogenesis, descendants of which mutated cell persist as additional cell line/s among the normal cells. The most common such finding is partial trisomy; to explain which we suppose that *anaphase lag* has occurred in an embryonic mitosis, producing trisomic and monosomic daughter cell lines by causing both chromatids of one member of one chromosome pair to be incorporated into the same daughter cell nucleus (cf. Cupisti *et al.*, 2003; Katz-Jaffe *et al.*, 2004), and leaving the other daughter cell missing one copy of that chromosome. However, we almost never find any cells with the autosomal monosomy corresponding to a discovered partial trisomy.

The mitotic error model for mosaicism generally accepted among clinical geneticists, the story usually told to medical students and to the parents of such patients, has become the standard answer by repetition alone. It is neither the only possible way to explain the routinely incomplete observations nor the most likely when all available evidence is considered together.

'Mosaics' identified clinically in this way are not rare among people with aneuploidy syndromes, particularly among those with relatively mild phenotypes. When we do undertake cytogenetic prenatal diagnosis by chorionic villus sampling, ~2% of such samples yield two cell lines, generally recognized as differing only because of an autosomal trisomy in some fraction of the cells (Viot, 2002). Most such cases are called examples of 'confined placental mosaicism', because we find the fetus itself normal at amniocentesis later in the pregnancy and normal at delivery. Unless the discovered 'mosaicism' involves a sex chromosome difference or at least two different chromosomes, no further examination is considered necessary (Falik-Borenstein *et al.*, 1994). There have been passing mentions of the possibility of a vanished twin as the source of the abnormal cells (Tharapel *et al.*, 1989; Kennerknecht *et al.*, 1991), but I find no published record of that prospect having been considered in any depth.

'Germline mosaicism' has become a routine explanation for certain apparent departures from Mendelian inheritance. When

a highly penetrant dominant disease allele disappears and reappears in a pedigree ('skips a generation'), when an autosomal dominant or X-linked recessive disorder appears as if by new mutation generating an abnormal allele not found in samples from either parent and then repeats in siblings (which a new mutation is highly unlikely to do), 'germline mosaicism' sometimes seems less improbable than the number or kinds of new mutations necessary to explain the observations (Cutler *et al.*, 2004; Ferreira *et al.*, 2004; Gloyn *et al.*, 2004). Germline mosaicism may be declared, to explain such discrepancies between siblings, and the mixed genotype parent is identified as such only later by further investigation (Mayr *et al.*, 1981; Yu *et al.*, 2002). The chimeric woman reported by Yu *et al.* (2002) would not have been discovered but for the level of genotyping involved in seeking a transplant donor, and the shock value of questionable maternity.

Finding chimeras

We do not expect to find chimeras because most of us are ignorant of their existence and the informed few just know they are too rare and bizarre to require consideration. We don't look for them because we don't expect to find them and we don't find them until we trip over evidence we cannot ignore. The human spontaneous chimeras identified as such to date comprise only the small fraction of all chimeras in the human population which we have been unable to ignore.

Most known chimeras have become known in one of two ways. There is blind chance, among people with unremarkable phenotypes, who are discovered in some genotyping situation to carry three or four, instead of one or two, alleles at multiple loci (Tippett, 1983; Bromilow and Duguid, 1991; Mifsud *et al.*, 1999; Drexler *et al.*, 2005). Routine blood-banking tests are nearly blind to small admixtures; unless there happen to be informative allele configurations in the subject's family for several of the routinely tested loci, and the minority genotype constitutes a substantial fraction of all cells examined, chimerism will generally not be discovered that way. One recent case was found when a surgical patient experienced acute intravascular hemolysis after transfusion of what more sensitive testing proved to be a unit of chimeric blood (Pruss *et al.*, 2003).

And there is sex. Most of the other chimeras we know about have been found because of a sex difference between the cell lines in a chimeric individual, manifested by anomalies of sexual anatomy or maturation or function, causing a search for an explanation for the odd sexual phenotype, leading to discovery of mixed cell lines (Verp *et al.*, 1992; Strain *et al.*, 1998).

Monochorionic boy-girl twins may be the most dramatic kind of mixed-sex anomaly (Souter *et al.*, 2003)—both sexes are no more 'normal' inside one chorion than inside one body. Whether or not we now know how they do that, we have every reason to believe they had to be together in a single mixed-sex embryonic cell mass when trophoblast differentiation occurred in the first few days of embryogenesis with both of them inside. Non-sexual developmental anomalies, if sufficiently visible, may also trigger appropriate investigation (Nyberg *et al.*, 1992). Predominance of sexual maldevelopment among discovered developmental anomalies is to be expected due to

the relatively benign nature of most sex development anomalies and the high level of interest it attracts. 'Boy or girl?' is still very often the first question society asks about each of its new members. My students are always astonished to learn how often the answer to that standard question is not perfectly clear and the harm that may come from forcing the issue.

Lessons from experimental chimeras

Many thousands of experimental chimeras have been generated for studies of embryogenesis and development (Gardner and Davies, 2000; Nagy and Rossant, 2001; Gardner, 2002; Tam and Rossant, 2003; Le Douarin, 2004). Transgenic animals, such important research tools in modern biotechnology, begin as chimeras, grown from embryos into which cells of a modified genotype have been inserted. In some of those, some of the extra cells will enter germ-line developmental pathways and produce gametes with the modified genotype. If the introduced mutation is compatible with viable development, this may allow for the breeding of whole-body transgenic organisms. Often, we learn at least as much from differences in development and functionality between the different cell types in the bodies of chimeric individuals. Those research chimeras would be useless for many of their intended purposes if chimerism tended to be homogeneous. It is characteristic of animal chimeras to be patchy, with one (piece of) tissue composed primarily of one cell type and the next of the other. Koopmans *et al.* (2005) show chimerism was never present in every organ examined from any single individual. It follows that failure to detect chimerism in blood or any other one particular sampled tissue is negligible evidence against the presence of chimerism in any other part of the same body. This is especially true when the tests in question are confined to cytogenetic analyses or routine blood antigen genotyping, or even a high-resolution genome scan performed on DNA from a single tissue, especially if signals from extra alleles are ignored as noise (if <30% of peak signal) or declared to have come from contaminated samples (if >30%) (cf. Ewen *et al.*, 2000).

Spontaneous chimeras are DZ twins (or mothers)

Some cases of human spontaneous chimerism may arise from embryonic or fetal cells colonizing a mother's body (Lo *et al.*, 1996; Reed *et al.*, 2004; Stevens *et al.*, 2004; Khosrotehrani and Bianchi, 2005; Koopmans *et al.*, 2005; Lambert *et al.*, 2005). This occurs, in some cases, with no pregnancy having survived to recognition. In all such cases, extra alleles must match the father of the conceptus from which the extra cells arose.

With the exception of this fetal-in-maternal chimerism, human spontaneous chimeras are products of DZ twinning events. DZ twinning is the only naturally-occurring human circumstance in which embryos with different genotypes are available to colonize one another. This is not the same as twin birth. Neither the delivery of the co-twin, nor any oddity of the placenta, nor any other evidence or suggestion of twinning is required. Chimerism arises from twin embryogenesis; it is not a function of gestation or delivery as twins.

If the genotypes of the cell lines in a human chimera are incompatible with belonging to siblings, then the chimerism is not spontaneous. Genotype data from parent/s or sibling/s may be required for a definitive answer to that question, which requirement might constitute a difficulty in the investigation of any case with no available first-degree relatives. However, even when the extra genotype clearly could be that of a sibling, if there are antibodies against the extra antigens, then the extra cell line producing those antigens was probably not present in the embryo before the establishment of immune self-tolerance. Cell lines in a spontaneous chimera will in general be cross-tolerant sibling lines.

Results

Chimeras are not rare

At upwards of one in 12, chimerism cannot be considered rare among liveborn DZ twins, and its occurrence in >20% of DZ triplet sets has to be called common (van Dijk *et al.*, 1996). The immunohistochemical method those workers used has a long history of reliable specificity and exquisite sensitivity (to detect one cell in 10 000 or more), but its use there was limited in scope. That work was performed under the assumption that chimerism in twins occurs exclusively by way of mixing of blood alone via placental anastomoses. Only blood was examined. All possibility of chimerism in other tissues was ignored. Their toolkit included fluorescent antibodies for a few marker antigens and their sample included only twins and triplets born alive as such. They could not have detected chimerism in any set the members of which were concordant for all of their marker antigens, nor in any individual whose second cell line had not survived to the time of testing, nor in any individual whose chimerism occurred only in tissues other than blood. Knowing parental genotypes would have given a better understanding of the relevant probabilities. The frequencies they report, astonishing as they are against the background of general understanding then and still, represent only a fraction of the chimerism among multiple conceptions.

'Kinds' of chimeras?

According to the literature, one might suppose that there are two or more 'kinds' of spontaneous human chimerism, differentiated by the imagined mechanisms of their origins. 'Dispermic', 'whole body', 'generalized' and 'tetragametic' are labels that have been used for cases acknowledged to have arisen from fusion of DZ twin embryos. Chimerism is said to be of this type when it is found in tissues other than blood or when adequate genotyping shows the twin cell lines to be discordant for paternal alleles (Osinska and Woloszyn, 1971; Dauber *et al.*, 1999; Wiley *et al.*, 2002). Otherwise, it is usually imagined to be of the supposedly more common 'twin' chimera type.

'Twin' chimeras are supposed to be chimeric in blood only, and to have become such by way of exchanging blood cells through anastomoses between their placental circulations (Angela *et al.*, 1976; Hosoi *et al.*, 1977; Pausch *et al.*, 1979; Bird *et al.*, 1980; Gilgenkrantz *et al.*, 1981). It has, however, become clear that chimeras among delivered DZ twins are far

more common (van Dijk *et al.*, 1996) than blood vessel anastomoses between dichorionic placentas (Robertson and Neer, 1983; Bjoro and Bjoro, 1985; Lage *et al.*, 1989; Benirschke, 1990, 1992, 1995; Machin *et al.*, 1995; Benirschke and Masliah, 2001; Foschini *et al.*, 2003). There are nowhere near enough anastomoses between dichorionic placentas to account for the observed frequency of chimeras. This can reasonably be considered to refute that traditional supposition. Reports of finding chimerism only in blood arise overwhelmingly from situations in which no tissue other than blood was examined.

Twin-to-mother-to-twin transfer?

An alternative explanation which we cannot presently exclude out-of-hand would be the transfer of blood between twins by way of the maternal circulation. We have known for a while, and made good use of the knowledge, that fetal cells are commonly found in the maternal circulation. We use fetal cells in maternal blood samples as substrate for non-invasive prenatal diagnoses. Detection in a mother's body of 'microchimerism' (small colonies of cells from her child or children), even decades after the corresponding pregnancy, and the prospect that those foreign cells might cause graft-versus-host 'autoimmune' disorder/s in the mother, has recently drawn attention (Stevens *et al.*, 2004; Khosrotehrani and Bianchi, 2005; Lambert *et al.*, 2005).

However, women with no history of pregnancy or transfusion are also commonly found to be chimeric in autopsy specimens of internal organs (tissue-specific cells, not just blood cells passing through, and in no case was the chimerism found in every organ examined from any given woman). The explanation offered as most likely was that the extra cells came from pregnancies that failed before clinical or maternal recognition (Koopmans *et al.*, 2005; cf. Boklage, 1990). That work was performed, for better understanding of transplant surgery results, by probing for cells that included Y-chromosome DNA sequences. For present purposes, clearly that approach ignores approximately half of all fetal-to-maternal-transfer chimerisms—in which the conceptuses providing colonizing cells were female. Furthermore, that approach allows for no proper further investigation of the prospect that some of the chimerism found in women with no history of pregnancy or transfusion may have arisen from their own embryogeneses rather than from unrecognized pregnancies. Extra alleles could and should be traced to determine whether the 'foreign' cells match mates or parents or siblings. (If they are products of conception, they must match the father of the conceptus. If arising from her own embryogenesis, they should match her parents or siblings. Only the latter should occur in virgin females.)

I have found the theoretical possibility mentioned, but have found no demonstration in human subjects that nucleated cells move from maternal to fetal blood with any frequency remotely comparable with that of fetal-to-maternal transfer. It should be easier, because maternal antigens in a fetus should encounter no immune resistance and would be expected to acquire permanently all benefits of self-tolerance when established by the child's immune system. I have been unable to find documentation of any significant frequency of permanent

maternal-to-fetal exchange of cells capable of ongoing development and the establishment of permanent colonies. Reed *et al.* (2004) have shown that maternal cells sometimes colonize a fetus, but they found it only in association with an HLA-DQ A1*0501 allele in the mother. Lo *et al.* (1996) found maternal DNA in almost half of their cord blood samples, but only at PCR sensitivity 1000-fold greater than that which sufficed to demonstrate all of the fetal-to-maternal transfers in their sample. This is not satisfying evidence that the average fetus routinely incorporates from the maternal circulation functional nucleated cells with the developmental potential to establish permanent chimerism.

DZ twins are not just womb mates

It is clear that most twin conceptions do not result in twin births. Survival of both members of a pair of twins from fertilization to term is rare (~1 in 50 in apparently optimal circumstances). There is a sole survivor from ~25% of twinning events and none from the rest. Sole survivors of twin conceptions are several times more common among live births than twins. By conservative estimate, sole survivors of multiple conceptions are at least as frequent as one live birth in eight (Boklage, 1990, 1995), roughly 10 times the frequency of twin pairs among all deliveries. Given that most spontaneous human chimeras discovered to date have been under the lifelong impression that they had always been singletons, there is no reason to suppose chimerism would be less frequent among sole survivors of DZ conceptions than it is among liveborn DZ twins (van Dijk *et al.*, 1996). We must infer that most chimeras are born single.

The traditional assertion that the excess prenatal mortality among twins is due to monozygotic (MZ) twins is gratuitous and wrong. Direct examination with good zygosity diagnosis shows that same sex DZ twins are at least as vulnerable to fetal and neonatal mortality as the MZs are (Boklage, 1985, 1987a).

The many ways in which twins of both zygosity differ in their development from singletons (Boklage, 2005a) do not result from gestation or delivery as twins, but from circumstances of embryogenesis peculiar to twinning—specifically from those parts of embryogenesis in which brain, craniofacial and behavioral asymmetries are established (Boklage, 1987b,c, 2005a; Gardner, 2001; Sudik *et al.*, 2001; Golubovsky, 2002, 2003a,b). DZ twins are developmentally at least as different from singletons as the MZs are, and in very much the same ways. The differences concentrate in embryogenic asymmetry variations of anterior midline structures.

Oddities of asymmetry development in twins have been falsely assumed to be routine and exclusive to the MZs from generations of folklore to the effect that MZ twins arise from some mechanical ‘splitting’ event whereby the embryo is torn in two and incipient structural asymmetries are disrupted and must find ways to realign if development is to continue (‘... what should have been the left side of Harry had to become the right side of George ...’). As witness, the enduring currency of the notion that same-sex twins discordant for handedness must be ‘late-splitting’ ‘mirror-image’ MZ twins (cf. Boklage, 1981; Derom *et al.*, 1996).

DZs, on the other hand, are supposed to come from separate and independent double (ovulation + fertilization + embryogenesis). According to that supposition, DZ twins have no reason to develop at all differently from singletons, especially in the establishment of structural and functional asymmetries in early embryogenesis and especially not to differ from singletons in the same ways that MZs do. But they do. They do just that, in every relevant way that they have been measured. DZ twins are not developmentally equivalent to singletons. The differences between DZ twins and singletons are very similar to the differences between MZ twins and singletons, and are not compatible with the expectations of independent double ovulation and independent embryogenesis as their origin (Harlap *et al.*, 1985; Boklage, 2005a).

Monochorionic male-female twins? That can't be right!

The male–female chimeric monochorionic DZ twins (MCOS-DZs) reported by Souter *et al.* (2003) are considered in the editorial of the same journal issue (Redline, 2003) as disproving dogma because they contradict the doctrine that monochorionicity is proof positive of monozygosity. Those presentations, however, leave a strong impression that they are seeing those MCOSDZs as a freakish exception that might almost rather prove the rule, caused perhaps by one or more of the ways that artificial reproductive technologies bring extra developmental vulnerabilities. But ... cells did it, cells never do anything they don't ‘know how’ to do, and cells don't know anything about the rules we have imagined for them. Dismissing or ignoring them is not okay. ‘How?’ seems likely to be important. ‘Dogma’ and ‘doctrine’ are not words too strong for this use. At the Fifth International Congress on Twin Studies in Amsterdam in 1986, a young physician from Glasgow tried to tell us about three monochorionic pairs among 12 in his sample, in whom he had found (with testing more extensive and more sensitive than the usual zygosity genotyping) discordant blood grouping markers suggesting dizygosity (Mortimer, 1987). The pillars of the Society came crashing down about his head. The tenor of the response from the floor was: ‘... of course, one must know, of course, that only monozygotic twins can be monochorionic. Results such as yours suggesting otherwise must have come from a very unreliable laboratory ...’

The foundations of the MC = MZ dogma as discussed in Redline (2003) are from Husby *et al.* (1991) and Vlietinck *et al.* (1988). Those studies were performed to test the applicability of Weinberg estimates of zygosity fraction against genotyped samples of twins. No twins who were identified in the studied birth records as monochorionic (these investigators did not attend the deliveries) were found to differ clearly at any of the loci tested, and they found no boy–girl twins recorded as monochorionic, which would have required further investigation if it were not summarily dismissed as obvious error.

Given that monochorionic twins apparently without exception do have placental anastomoses through which they exchange blood, concordance for the handful of blood antigen markers used to test zygosity in these samples cannot be considered overwhelming evidence. Souter *et al.* (2003) reported that the initial genotyping of the MCOSDZ pair they reported

was consistent with monozygosity. Using a nearly identical panel of markers in an experimental control not mentioned in Husby *et al.* (1991) or Vlietinck *et al.* (1988), Nylander and colleagues found genotypes concordant for all tested markers, consistent with criteria for confident diagnosis of monozygosity, in approximately one quarter of the boy–girl pairs in their samples. They ‘corrected’ their results from the same-sex pairs accordingly (Nylander, 1974; Nylander and Corney, 1977) and called the corrected results consistent with Weinberg method expectations without addressing the implication of reduced polymorphism among the parents of twins.

A number of other MCDZ pairs have been reported (Nylander and Osunkoya, 1970; Iselius *et al.*, 1979; Bieber *et al.*, 1981; Vietor *et al.*, 2000; Quintero *et al.*, 2003; Williams *et al.*, 2004; Yoon *et al.*, 2005), plus the recent cluster of six such pairs reported by Miura and Niikawa (2005). The MC pair reported in Bieber *et al.* (1981) was investigated because one member was acardiac; extensive genetic differences proved dizygosity. The MC twins reported in Yoon *et al.* (2005) were investigated because of visible discordance for what proved to be Beckwith–Wiedemann syndrome. They were found to be DZ, discordant also for Klinefelter syndrome and several unlinked marker loci. All of the others in these references are boy–girl pairs, without which unignorable oddity monochorionicity would have been unremarkable and the possibility that they were dizygotic would almost certainly not have been investigated.

The Beckwith–Wiedemann syndrome, by the way, is reported to be excessively frequent in monozygotic twin pairs, with an excess of female pairs, and almost always discordant (Weksberg *et al.*, 2002; Bestor, 2003). The excess of twin pairs associated with Beckwith–Wiedemann has been identified as monozygotic, in spite of substantially discordant phenotypes ... generally because of sex-concordant monochorionicity. It is not clear that the level of genotyping capable of discovering chimeric dizygosity was performed in any of the reported cases.

There is ample reason to suppose, and to test the prospect carefully, that monochorionic DZ twins are also rather more frequent than finding them is.

Two into one, and back

In thousands of experiments in which all or part of one experimental mammalian embryo has been put inside another one, of same or different genotype (or sex or strain or species), the result is not twinning but single chimeric offspring or embryo failure (cf. Gardner and Davies, 2000; Gardner, 2002). The development of separate twin bodies from a single embryonic cell mass (regardless of the number of genotypes among those cells) requires the cellular behaviour of a monozygotic twinning event ... subsets of the cells in the mass must establish two distinct systems of body symmetries, two sets of head–tail, back–belly and left–right axes.

This is all there is to ‘splitting’. In the first few cell divisions, molecular decisions are made about where the head and the tail are supposed to go, who gets to be back and which has to be belly, and which cell will get the transcription factor

subsystem that will determine that its progeny will later migrate into the gonadal ridges to induce the differentiation of the gonads and become the gametes. Unless something is badly wrong, the entire three-dimensional armature is microscopically visible as soon as the location of the prochordal plate and/or the primitive streak becomes apparent to mark anterior versus posterior and leave left–right no choice because dorsal–ventral has already been clear for a few days. All the axes are quite clear by the sixth or seventh day because it takes a while for the cells to show up in their proper places after the organizing decisions are made. This is only a day or so after the zona comes off, so all those decisions must normally be made while still inside the zona. The zona pellucida is elastic. It’s tight in there. No room in there for anything that could be visualized as a ‘split’. No ripping. No tearing. No child’s hair to tie the one embryo almost in two à la Spemann. The cells just set themselves up in two patterns. As we traditionally interpret the meaning of chorionicity: if such twins are to be dichorionic (apparently, but hardly proven to be, the more common outcome), the separate systems of body axes must be established within the first 1–3 days post-fertilization. A few hours less quickly, and they assume the extra gestational hazards of monochorionicity.

We have no evidence of any constraint on the final allotment, between the twins, of cells of the different genotypes. The results in van Dijk *et al.* (1996), limited to what can be understood from blood alone, show some very small numbers of cells of the co-twin’s genotype and some quite substantial fractions, some reciprocal exchanges and some apparently one-way.

On the fusion of male + female embryos

The normal excess of males in human births in spite of most reports showing excess male losses throughout pregnancy apparently can be explained by observations that a paternally imprinted X-chromosome (normally present only in female embryos) substantially slows female embryogenesis (Boklage, 2005b). Much faster early development in male versus female embryos would seem likely to predict a predominance of male phenotypes for mixed-sex chimeric individuals, and might be expected to suppress (below the theoretical binomial half) the frequency of live-born chimeric twin pairs appearing as normal boy and normal girl.

Same-sex pairs are found in excess among delivered DZs (James, 1992)—in spite of prenatal losses concentrated in same-sex pairs (Rydstroem and Heraib, 2001)—among which SSDZs are at least as vulnerable as MZs (Boklage, 1985, 1987a). This follows the pattern behind the ‘secondary sex ratio’ (Boklage, 2005b) and suggests the parallel possibility that the excess of SSDZ pairs at birth, in spite of excess losses among SS pairs throughout pregnancy beyond embryogenesis, may be established by excessive failure of OSDZ pairs in embryogenesis, before pregnancy recognition. Overgrowth of male cells in mixed-sex embryos could cause OS chimeric embryos to appear later in pregnancy as male twins. Most sex-chimeric mice become fertile males (Tarkowski, 1998).

The members of normal, ordinary, dichorionic live born male–female pairs clearly have not developed independently.

They do not have the normal statistically obvious sex differences in craniofacial development found in singletons and members of same-sex twin pairs (Boklage, 1984), and both members of male-female pairs show fetal and neonatal mortality that is significantly lower than their counterparts in same-sex pairs (Boklage, 1985, 1987a).

The excess of males in human births appears due to a paternally imprinted X-chromosome retarding female embryogenesis relative to that of males (Boklage, 2005b). The male excess at birth is lower for fathers of African descent than for white European fathers, and higher for Asian fathers. This could mean that the more permissive the paternal X-imprint, the more females, the more twins, and the more male-female twins reach term birth. Also differing over these populations in the same order: average female age at menarche, at first birth and at last birth. Also in the same order, the earlier the trophoblast differentiates, and the greater is the fraction of dichorionic pairs among same-sex pairs and the lower the fraction male among monochorionic pairs and still more so among monoamniotic pairs. The more permissive the paternal X-imprint, the faster apparently moves every aspect of reproduction in females.

Miura and Niikawa (2005) have supposed that artificial reproduction technologies (ART) might be promoting chimerism because all the MCOSDZ pairs they discovered were products of ART procedures in Japan. Given that they would not have found the chimerism in any of those cases had not monochorionic boy-girl twins attracted their closer attention, given that natural Japanese twins are known for their low frequency of OS pairs, given the survival issues surrounding all twins, and given the male > female embryonic growth rate discrepancy, I propose that ART need not increase the probability of chimerism in general as suggested by Miura and Niikawa (2005), but instead need only make rates of male and female embryogenesis more equal by an epigenetic effect, such that the female cells in mixed-sex chimeras would be less likely to be outgrown or pushed aside into an ineffective minority—the better to see ‘normal’ boy + ‘normal’ girl twins at birth. There is good and growing evidence that ART protocols in current use are associated with disorders of imprinting (Paoloni-Giacobino and Chaillet, 2004; Gardner and Lane, 2005; Maher, 2005; Shiota and Yamada, 2005). Normal sex-dependent differences in speed of human embryogenesis are reported absent in IVF embryos (Dumoulin *et al.*, 2005).

Discussion

If natural DZ twins must in general arise from independent double ovulations and independent embryogeneses, then spontaneous chimerism should probably be even more rare than it has been imagined to be.

The evidence, however, shows that chimerism is not at all rare and that it must arise primarily from fusion of DZ embryos—an outcome very difficult to explain beginning from independent double ovulation and embryogenesis. What we know about the chimeras we have found and the ways we have found them demands the inference that those human chimeras who have been identified as such constitute a small minority,

and that the undiscovered majority are normal people whose chimerism will most probably never be discovered. Human spontaneous chimeras are common; only those identified as such are rare. Chimeric individuals whose bodies are composed of two normal cell lines, or in whose bodies cells of an abnormal line constitute an ineffective minority or exist only in tissues unlikely to be sampled, must constitute the majority of all chimeras and draw no special attention.

Dichorionic twin placentas grow together (‘fuse’) about half the time, but anastomoses between them are very rare in either zygosity. Spontaneous chimerism is not rare; therefore, placental anastomosis cannot be the way most chimerism happens. If we should wish to maintain the tradition that chimerism results overwhelmingly from mixing of blood alone against the evidence that chimerism is far more common than placental anastomoses between DZ twins, then there is a need for exciting new evidence showing that exchange of pluripotent cells between DZ twins can and does occur quite commonly by way of the maternal circulation. Until such evidence can be gathered, I must infer that spontaneous human chimeras arise primarily from fusion of DZ twin embryos and seldom if ever from fusion of their placental circulations. Many cases of chimerism can be explained only by fusion of DZ embryos, but I can find no case proven to have arisen from exchange of blood alone between DZ co-twins via either placental anastomoses or passage from one twin to the other through the maternal circulation. Chimerism of blood alone is reported overwhelmingly from circumstances in which only blood was examined.

It was suggested that I should consider ‘stress effects related to having multiple embryos in a single womb’ as a possible ‘cause of characteristics specific to both MZ and DZ twins’—rather than sharing a history of deriving two body symmetries from a single embryonic cell mass. The differences at issue here—in a/symmetry-dependent development of neural tube, cardiac tubes, craniofacial structures and brain function—all depend upon cellular/molecular axis-definition processes which must occur in the first few days, while the conceptus is microscopic and probably before even hormonal communication with the mother. Any stresses at issue here seem certain to be internal to the embryogenic process, and it seems important that the outcomes do not differ by zygosity.

The question arises whether DZ twins from independent double ovulation might become monochorionic without spending time together in a single cell mass, perhaps by being close enough at blastogenesis that their respective chorion-precursor trophoblasts might fuse around them. Because blastogenesis and trophoblast differentiation normally happen inside the zona pellucida, premature removal or fusion of the two zonae would be topologically essential to allow cells of the respective trophoblasts even to touch. To arrange such events for experimental purposes, as mentioned above, requires removal of the zonae. In general, the two inner cell masses coalesce as well. Roughly half of all pairs of dichorionic placentas, regardless of zygosity, appear as fused later in pregnancy. Recognition of their dichorionicity in spite of such fusion is not trivial, but routine.

Opposite-sex twins are roughly half of all DZ twins and roughly a third of all live born white European twins. The fraction

of all twins who are OS is larger among live born twins of African ancestry than among white European twins, and smaller among live born twins of Asian ancestry. Except for certain very rare anomalies, opposite-sex twins are dizygotic. (The assumption that OS twins should be exactly half of all live born DZ twins is an approximation, crude at best for any group other than healthy white European twins. The other standard assumption required for any faith in the utility of the Weinberg method of estimating zygosity fractions, namely that OS twins are developmentally equivalent to SS-DZ twins, and thus developmentally representative of all DZ twins, is nonsense.)

Monochorionic twins as a group have more problems than dichorionic twins as a group, but they constitute about half of live born MZ twins of African ancestry (Nylander, 1974; Nylander and Corney, 1977), about two-thirds of live born white European monozygotic twins (Vlietinck *et al.*, 1988; Husby *et al.*, 1991) and >80% (Yoshida and Soma, 1984) of Japanese MZs. (The old Weinberg-based assertion that only DZ twinning varies over subpopulations, while MZ twinning is constant, has persisted in spite of these variations in the biology of MZ twinning.) Monochorionicity has been considered certain proof of monozygosity. Rarely, monochorionic twins are of opposite sex because one has normal 46,XY cells and his twin is a 45,X Turner syndrome female missing the second sex chromosome in all of her cells (extrapolation assumed from a non-mosaic blood karyotype). We call those ‘heterokaryotic’, monozygotic twins. They are supposed to have arisen from a single zygote, but they have different karyotypes due to anomalous X,Y chromosome segregation in embryogenesis (that would be textbook mosaicism followed by twinning—one might wonder whether there are in fact no autosomal counterparts). 45X,46XY–heterokaryotic MZ twins cause us no theoretical anxiety as long as we can believe that the 45,X female has no 46,XX cells.

Beware of the dogma

Twins who are both opposite-sex (46,XX and 46,XY) and monochorionic raise very different issues. It is not supposed to be possible. It does, however, occur. Therefore, it can. It can occur only by way of embryo fusion. That is what makes it so ‘wrong’. The MC = MZ doctrine is only a corollary of an older and deeper dogma at issue in these considerations—the ‘common knowledge’ that DZ twins just do arise from double ovulation (Boklage, 2005a). Only because of that article of faith is the idea of monochorionic, dizygotic twins any sort of surprise in the first place. The same idea is all that stands in the way of understanding chimerism as primarily the result of DZ twin embryo fusion, having little or nothing to do with exchanging only blood through placental anastomoses. Monochorionic DZ pairs particularly and obviously, and spontaneous chimerism in general, imply and require that some fraction of DZ twins have spent at least part of their embryonic lives in a single cell mass. This is extremely unlikely in the shadow of the DZ double ovulation dogma, but not so much if we can drag it out from under there into better light (Boklage, 1987a,b, 2005a). Spontaneous chimeras via DZ embryo fusion, and especially MCDZs,

satisfy predictions of an alternate model for the cellular origin of DZ twins—which arises from a list of observations that the hypothesis of independent double ovulation cannot satisfy.

Mechanism(s)

Plausible cellular alternatives to independent double ovulations as source of DZ twinning would have them arising from daughter cells of single secondary oocytes divided symmetrically before sperm entry (‘tertiary oocyte twins’ (Boklage, 1987b,c), often called ‘polar body twins’), or those same two half-genomes in an as-yet-undivided secondary oocyte (Golubovsky, 2002,2003a,b; St Clair and Golubovsky, 2002). Some find it easier to think of this as a ‘rescue’ pathway for over-ripe or otherwise compromised oocytes (cf. Bomsel-Helmreich and Papiernik-Berkhauer, 1976; Harlap *et al.*, 1985; Boklage, 1987b,c). In all of the possible mechanisms, there must be two paternal pronuclei (generally from two sperm cells, but diploid sperm are apparently not yet conclusively ruled out), achieving syngamy with two maternal pronuclei arising from the second meiotic division of the secondary oocyte nucleus, one of which ‘should have been’ discarded in the second polar body. The maternal pronuclei may be in one cell with an unfinished second meiotic division, or two (tertiary oocytes) after a symmetrical second meiotic division. All variations have the final common expectation of two syngamies producing two zygotes inside a single zona pellucida—indistinguishable from any other two-cell embryo except that those first two cells are of different genotypes. The existence of MCDZ twins requires that it be possible; the apparent origins and distribution of chimerism require that it be frequent.

Assuming that only mothers could influence any probability of twinning by double ovulation, we must suppose that the well-documented paternal effects on probability of DZ twinning (Carmelli *et al.*, 1981; Sathananthan *et al.*, 2001; Golubovsky, 2002; St Clair and Golubovsky, 2002; cf. Tesarik, 2005) are exerted through monovular DZ twinning. The frequency of triploidy shows an ample supply of doubled contributions from both maternal and paternal sources (McFadden and Langlois, 2000; Zaragoza *et al.*, 2000; McFadden *et al.*, 2002; Golubovsky, 2003a,b). Other major pieces of this puzzle include: (i) suspension of the second meiotic division pending sperm penetration; (ii) the dependency of syngamy and early embryogenic cell division on the centrosomal material and centriole/s provided by the sperm (van Blerkom *et al.*, 1995; Palermo *et al.*, 1997; Sathananthan, 1997; Sutovsky and Schatten, 2000); (iii) the need for the oocyte to conduct a major rearrangement of the sperm chromatin to transform it into a functional paternal pronucleus (Gioia *et al.*, 2005); and (iv) other changes in the oocyte after ovulation (reviewed in Boklage, 1987b,c). This system of interactive processes required to complete fertilization provides a plausible focus for questions of paternal influence and monovular DZ twinning.

For embryogeneses beginning from a configuration of two zygotes in a single zona, a single chimeric offspring would seem at least as likely as the formation of separate twin bodies. If separation is achieved (requiring the same cellular behaviours as monozygotic twinning), so that concurrent embryogeneses

may proceed in parallel beyond that intersection, the likelihood that the two embryos would carry souvenir cells of each other's genotype seems high.

The existence of monochorionic dizygotic twins provides an unavoidable lesson: twin zygotes, same sex or different, do at least sometimes form a single mixed embryo from which they may emerge as viable twins, often carrying samples of cells from each other mixed in as they build their separate bodies. In such chimeric embryos, spontaneous internal definition of two body symmetries occurs, perhaps most commonly before (dichorionic) but at least sometimes after (monochorionic) cellular commitment to the differentiation of the trophoblast. We have no evidence as to the relative frequencies of those two possibilities. To date, the number of discovered monochorionic DZs is small, but not particularly small compared with the probability of finding them without looking. Any prospect a fused embryo will have for development to live birth as two separate individuals requires the very same cellular event as monozygotic twinning, namely, to create two systems of body symmetry axes inside a single mass of cells, so that they can begin and continue to grow out as two bodies. The twin bodies that may be built upon those cellular/molecular armatures are dizygotic but not independent—they have been at least temporarily within the same one embryonic cell mass. According to the evidence accumulated here, this occurs with much greater frequency than previously imagined, with many more cases undiscovered for want of asking the necessary questions. Unless exchange of pluripotent stem cells between twin fetuses through the maternal circulation can be shown to be routine, such an outcome seems highly improbable for twin embryos from independent oocytes. Chimerism, would, however, be quite ordinary for twin embryos that begin development within a single zona pellucida.

Immunology

Another prediction may be in order. There is lore to the effect that co-twins diagnosed as monozygotic should be perfect tissue transplant donors for each other, and that DZ co-twins should be no better than any other siblings, with only ~25% chance of matching for the primary transplant-compatibility genes. While grafts or transplants between twins who are supposed to be 'identical' do not always take without some immunosuppression (Golembe *et al.*, 1979; Hinterberger *et al.*, 1997), I have found no published evidence that transplant efforts between HLA-non-identical DZ twins have been made on many occasions and constantly failed. Perhaps it has been faithfully assumed that DZ co-twins would be as limited as singleton siblings as source of necessary tissue transplants ... assumptions of that sort are certainly common around twins. If DZ twins are reciprocally chimeric as often as they have been reported to be (van Dijk *et al.*, 1996), let alone as much more often than that as I am arguing here, and if that chimerism has been in place since embryogenesis—before and during their immune systems' establishment of self-tolerance, then it seems likely that somewhat more than the HLA-identical ~25% of DZ co-twins might in fact be reciprocally suitable transplant partners (Nylander, 1974; Summers and Shelton, 1985). Such

DZ transplant tolerance will occasionally be unidirectional—when only one of the co-twins carries cells of the other's genotype, the single-genotype twin may be expected to reject tissue from the chimeric co-twin. This latter prospect, in turn, might explain some of the transplant difficulties between twins thought to be 'identical' because of sex-concordance and monochorionicity.

Embryogenesis of anterior midline functional asymmetries

The human brain appears to surpass substantially any other kind in the extent and importance of left-right asymmetry in its functionalities. Left-handers plus the ambidextrous comprise a minority variously estimated at ~10% of the population. They differ from the 'strictly' right-handed folks in many ways. According to most genetic models still given any consideration, these 'nonrighthanders' (NRH) constitute a random half of a minority whose members lack the cellular or molecular determinants required to establish the normal/majority human brain function asymmetry. Twins and their parents and siblings (very importantly, of both zygosity equally, and independently of chorionicity among the MZs) have a clear excess frequency of NRH (Boklage, 1981, 1987b,c; Derom *et al.*, 1996). The malformations that are excessive among twins (neural tube defects, orofacial clefts and congenital heart defects most prominently—all midline/fusion anomalies) are excessive also among first-degree relatives of twins, and all have strong associations with NRH among singleton victims and their first-degree relatives as well. Clearly, neither twin gestation nor twin birth, nothing about twinship beyond associated heritable variations in embryogenesis, causes any of these developmental asymmetry anomalies, because their single born parents and siblings and offspring and unrelated singletons show the same associations. In most of these relationships, there is no zygosity difference. Where there is a zygosity difference, the relationships tend to be stronger among DZ than among MZ twins (e.g. Klaning *et al.*, 2002). This is strongly contrary to the old notions that anomalies such as these belong strictly to the MZs because of their exclusive involvement with some odd sort of embryogenesis. There is no escape from the inference that DZ embryogenesis is more or less exactly as odd as that of the MZs, and no reason to suppose it could get that way beginning with independent double ovulation.

Variations such as these in brain function asymmetry are associated with virtually every oddity of human mental or behavioural development and function. The exact cellular and molecular processes of defining two systems of brain and body symmetry axes from within a single embryonic cell mass, and the results thereof, might reasonably be imagined to differ from the usual embryogenic protocol of defining only one developmental armature per embryonic cell mass. Whatever that system of differences may be, this phase of embryogenesis must be where the symmetry development differences originate between singletons and twins. The developmental differences in embryogenic asymmetry determination between DZ twins and singletons as groups are not occasional or accidental: groupings calculated from patterns of craniofacial development are coherent and highly statistically significant (Boklage,

1984) with negligible overlap. These results represent symmetry determination specific to the head and the neural crest, involved with midline fusion, craniofacial, brain and behavioural development (Boklage, 1984, 1987bc, 2005; Klaning *et al.*, 2002; Klar, 2005; Mitchell and Crow, 2005). The results give no reason to suppose that only some DZs, any less than all, are developmentally different from singletons. That system of asymmetry differentiations might or might not differ from any other such processes (Levin, 2004, 2005) that appear later and might not involve neural crest mesenchyme. DZ twins and MZ twins are equally different from singletons in patterns of craniofacial asymmetry development. Twin heads are built differently from singleton heads. In the major components of that system of differences, MZ and DZ twins differ from singletons equally. Any part of the MZ developmental oddities arising from their having developed from two different body symmetry systems in a single cell mass happens to DZ embryos in the same or very similar ways.

I am convinced that the normal process of establishing the body's axes of asymmetry is initiated primarily by a cascade of epigenetic mechanisms anchored in the fundamental asymmetry of the DNA. A sizable body of excellent work (cf. Levin's reviews, 2004, 2005) has demonstrated cascades of transcription control mechanisms that contribute to defining the embryonic origins of structural and functional asymmetries. All such systems reported to date begin with a signal that is already reliably asymmetric. They cannot therefore be considered to have answered the fundamental question, but only to have pushed the question back a little. How are we to suppose that the gene encoding the first transcription factor signal in the cascade knows how to kick things off by always first producing its product from cells on one side of the embryo and always the same side? (Please note that we can only say which side if we know which side is which, and I must insist that the cells had to 'know' that before they could set themselves up so that we could see it.) I have suggested DNA as the source because it brings the necessary reliable asymmetry forward from the beginning, and cells of every living thing appropriately questioned have demonstrated ways to know the difference between old strand and new, leading strand and trailing strand (Pierucci and Zuchowski, 1973; Dalgaard and Klar, 2001a,b; Klar, 2004a) for their use in allocating the modifications that constitute their epigenetic programming (Santos and Dean, 2004).

From such a perspective, it seems that an embryo with cells of two genotypes (and epigenotypes) would be more likely than a single-genotype embryo to establish two systems of embryogenic body axes. This would be entirely consistent with, and might help to explain, the fact that all reproductive procedures that involve artificially induced ovulation (which necessarily and always departs from natural oocyte maturation) increase frequencies of both polyzygotic and apparently monozygotic twinning events (Derom *et al.*, 1987; Hankins and Saade, 2005).

The presence of two distinct and potentially incompatible genomes and epigenomes in one embryo, each working from its own logic to establish its own version of structures around and across the midline, might interfere with normal determination of functional asymmetries. Most cases of functional asymmetries

of body and brain differing from those in the normally lateralized majority could find their explanation in twin embryogenesis or chimerism and associated anomalies of epigenetic control. That grouping will include, and may help to explain, nearly every individual any of whose functional asymmetries of brain or body differ from those of the majority, including but probably not limited to natural NRHs, most cases of midline fusion malformations, most cases of functional psychosis, alcoholism, or spontaneous seizure disorders, and most cases of genitalia-discordant sexual orientation (Klar, 2004b).

The oddities by which we may rarely discover spontaneous chimerism are not required for its occurrence, and there is no reason to imagine that spontaneous chimerism is a quantum-mechanical event that owes its existence to being observed. In fact, a substantial fraction of us are built of cells that grew from zygotes that might have become two people, with different genomes and different epigenomes, different (and potentially conflicting) systems of genes and gene expression patterns responsible for directing the construction and function of bodies and brains. And, with only those exceptions in which one or both of the cell lines causes a visible problem, chimerism in general makes no difference we now know how to interpret as such, and no one need ever know.

The fraction of the population who are chimeric might be as high as 10% or more. Conservatively estimated, at least one live birth in eight is a product of a twin conception, the majority of which bring with them to delivery neither a co-twin nor any other overt evidence of their twin history (Boklage, 1990,1995).

The capacity for reflection provided by the structural and functional duality of the human mind-brain is arguably its greatest distinction from, and its greatest evolutionary advantage over, the brain of any other organism. Reflection is the mental substrate of self-awareness, and of the creative power of experiment and comparison. It provides the survival-value luxuries of the products of those processes and the safety of offline rehearsal. The mechanisms underlying the development of the necessary dual functionalities are closely involved at cellular and molecular levels with the mechanisms and consequences of twinning, which must be understood to include chimerism.

Summary

(i) Human spontaneous chimerism is common—plausibly of the order of 10% of the population.

(ii) Most spontaneous chimeras are DZ twins who have exchanged cells as embryos. Some are mothers colonized by cells from offspring *in utero*—some of whom never had a recognized pregnancy.

(iii) Most chimeras, like most twins, are born single.

(iv) Chimerism rarely if ever arises from placental anastomoses.

(v) Twin embryogenesis is associated with anomalies of midline fusion asymmetries, affecting twins of both zygosity equally and in the same ways.

(vi) Midline asymmetries of nervous system, face and heart are established in the same first few cell divisions of embryogenesis in which twinning occurs.

- (vii) Every anomaly attributed to odd embryogenesis in MZ twins happens with equal or greater frequency in DZ twins.
- (viii) DZ embryogenesis is at least as odd as that of MZ twins.
- (ix) There is no evidence that any pair of natural DZ twins ever came from double ovulation.
- (x) DZ embryogenesis happens the same way as MZ embryogenesis—defining and growing out two body symmetries from a single mass of cells.
- (xi) Some DZ twins are monochorionic and some monochorionic twins are DZ; the same could be true of monoamniotic twins.
- (xii) Chimeras, like other DZ twins, arise from monovular embryos
- (xiii) Many non-HLA-identical DZ twins will be mutually excellent tissue transplant donors; sometimes, it will only work one-way.
- (xiv) Many ‘mosaic’ cell lines will be found to be chimeric if properly tested.
- (xv) Autopsy specimens are a reasonable place to look for chimerism in tissues other than blood.

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