(1)

主論文

Developmental alterations of α-fetoprotein sugar chain in amniotic fluids analyzed by lectin affinity electrophoresis

Lectin-affinity electrophoretic analysis of amniotic fluid AFP is useful for the evaluation of developmental state of fetus with respect to AFP sugar chain.

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Abstract

Affinity electrophoresis of human α-fetoprotein (AFP) in amniotic fluids from pregnant women between 6 to 42 weeks of gestation and in serum of yolk sac tumor was performed with concanavalin A (Con A), lentil lectin (LCA), Erythroagglutinating phytohemagglutinin (E-PHA) and *Allomyrina dichotoma* lectin (allo A). Separated AFP bands were detected by sensitive antibody-affinity blotting. In the first trimester, amniotic fluid AFP showed increased proportions of Con A-nonreacting AFP (AFP-C1) (14) and LCA weakly-reacting AFP (AFP-L2) (14) as reported(4-6). Additionally, high percentages of E-PHA strongly-reacting AFP (AFP-P5) (14) and E-PHA-reacting AFP (AFP-P4) (14) were observed. E-PHA-nonreacting AFP (AFP-P1) (14), E-PHA weakly-reacting AFP (AFP-P3f) (14), allo A-nonreacting AFP (AFP-A1) (14) and asialo-AFP, AFP-A1s(14), were present only in amniotic fluids of 6 to 17 weeks of gestation. With advancing gestation, proportions of AFP-C1, AFP-P4 and AFP-P5 decreased and AFP-L2, AFP-P3f, AFP-A1 and AFP-A1s disappeared by the end of 18 weeks. The glycoforms of serum AFP of yolk sac tumor resembled those of amniotic fluid AFP in early gestational stages.

Key words: α-fetoprotein, affinity electrophoresis, lectin, amniotic fluid, yolk sac tumor

Human α-fetoprotein (AFP) is an oncofetal glycoprotein with a molecular weight of approximately 69,000 with 4% carbohydrate. The carbohydrate structure of AFP is principally biantennary complex-type oligosaccharides linked to asparagine. The main carbohydrate components are *N* -acetylglucosamine, mannose, galactose and sialic acid (1). The sugar chain heterogeneity or glycoforms of human AFP in fetal sera was first demonstrated by Smith and Kelleher (2) in 1973 using lectin affinity chromatography. Amniotic fluids have high levels of AFP and their sugar chain alteration has been extensively studied by Kelleher et al. (3), Mackiewicz et al. (4), Ishiguro et al. (5,6), Nørgaard-Pedersen et al. (7) and Toftager-Larsen et al (8-10). They analyzed AFP glycoforms by column affinity chromatography (3), tandem crossed immunoelectrophoresis (4) and crossed-line affinity immunoelectrophoresis (5-9) using concanavalin A (Con A) (1-5,9-11), *Lens culinaris* agglutinin (LCA) (1,4,5,9,10), Erythro-agglutinating phyto-hemagglutinin (E-PHA) (5,10) and *Ricinus communis* agglutinin I (5). In their studies, it was not necessarily easy to analyze the glycosylation of low levels of AFP in early gestational stages because of the limited sensitivity of their method in detecting separated AFP glycoforms. Taketa et al. (12,13) developed a sensitive method of antibody-affinity blotting for the detection of AFP glycoforms separated by lectin affinity electrophoresis. In the present study, we adopted the sensitive method of antibody-affinity blotting coupled with immunoenzymatic amplification for the detection of separated AFP bands in order to analyze samples with low levels of AFP. This allowed us to see minor sugar chain alterations, which were not analyzed in the above mentioned studies. In addition to commonly used lectins such as Con A or LCA, we used E-PHA and *Allomyrina dichotoma* lectin (allo A).

Materials and methods

Materials

After informed consent was obtained, amniotic fluids were obtained at the time of dilatation and curettage, amniocentesis, caesarean section or vaginal delivery from 14 normal pregnant women between 6 to 42 weeks of gestation. Serum from a patient with yolk sac tumor was also obtained. These samples were kept frozen at -25 ℃ until analysis.

Glycoforms of amniotic AFP were separated by affinity electrophoresis with lectins and separated AFP bands were detected by antibody-affinity blotting followed by immuno-enzymatic amplification (12,13) as follows:

Lectin-Affinity Electrophoresis

Samples were diluted with barbital/barbital-Na buffer (ionic strength 0.025, pH 8.6) to give an AFP concentration of 100 ng/ml. Affinity electrophoresis was carried out by applying 4 μl of samples to troughs in agarose gels (Litex Laboratory, Glostrup, Denmark) 1.0 mm thick containing 1.0 mg/ml Con-A (Pharmacia Fine Chemicals, Uppsala), 0.2 mg/ml LCA (Seikagaku Co., Tokyo), 0.3 mg/ml E-PHA (Seikagaku Co.) or 0.3 mg/ml allo A (Cosmo Bio Co., Tokyo). Electrophoresis was run at 10 ℃ by giving a constant voltage of 15 V/cm until free bromophenol blue migrated 55 mm from the origin.

Antibody-Affinity Blotting

Separated AFP bands were detected by the antibody-affinity blotting. The gels were overlaid with antibody-precoated nitrocellulose (NC) membranes and with filter paper pads to transfer the AFP bands by capillary blotting to the NC membranes. NC membranes were precoated with mouse anti-human AFP monoclonal antibody (NB-011, Nippon Bio Test Laboratories, Tokyo) diluted in Tris-buffered saline (TBS) (20 mmol/L

Tris-HCl, pH 7.5, 500 mmol/L NaCl) at a concentration of 100mg/ml.

Immunoenzymatic Amplification

Enzyme immunodetection was performed by specifically transferring AFP to the NC membranes and transferred AFP was vizualized by treating the transfers with rabbit anti-human AFP antibodies (DAKO, Copenhagen) diluted 1,000-folds with 1.0% gelatin-containing TBS, followed by horseradish peroxidase-labeled goat antibodies against rabbit IgG (Bio-Rad Laboratories, Richmond) diluted 1,000-folds with 1.0% gelatin-containing TBS. Washing of the membranes was made twice for 5 minutes for every step of treatments.

The antibody-treated membranes were stained by the tetrazolium method (13) for peroxidase reaction.

Intensities of stained AFP bands were quantitated by densitometric scanning with a Scanning Densitometer Model 1650 (Bio-Rad Laboratories) after immersing the developed and dried NC membranes in decalin. The intensities of AFP bands were expressed as percentages of total AFP bands after identifying the bands by the nomenclature system of Taketa et al. (14) namely, major AFP bands were numbered consecutively from the anode, giving the lowest Arabic numeral 1 to the most anodal band, and the numerals were suffixed to capitalized initial letters of the lectin used. Minor or infrequently appearing bands were identified by adding "s" for slow-migrating and "f" for fast-migrating bands relative to the major bands.

Results

Patterns of AFP bands separated by affinity electrophoresis with four

lectins for amniotic fluids and serum from a patient with yolk sac tumor are shown in Fig.1. Con A separated AFP into two bands, Con A- nonreactive AFP-C1 and reactive AFP-C2, the later being slightly broader. LCA separated AFP into three bands, LCA-nonreactive AFP-L1, weakly-reactive AFP-L2 and strongly-reactive AFP-L3. E-PHA separated AFP into five sharper bands, E-PHA-nonreactive AFP-P1, less weakly-reactive AFP-P2, weakly-reactive AFP-P3f, less strongly-reactive AFP-P4 and strongly-reactive AFP-P5. AFP-P3, which migrates in between AFP-P3f and AFP-P4, was absent. Allo A separates AFP into three bands, allo A-nonreactive AFP-A1, weakly-reactive AFP-A1s and strongly-reactive AFP-A3, AFP-A3 being the major band.

The results of densitometric scanning of these AFP bands from amniotic samples are summarized in Figs. 2～5. Reproducibility of the results was confirmed by multiple assays on 24 other available samples. The proportions of AFP-C1,which are plotted against the gestational week in Fig. 2, decreased from 85% in the 6th gestational week to 15% in the 16th～17th gestational weeks and were hardly recognized at near term after 36th gestational week. The proportions of AFP-L2 and AFP-L3 are plotted against the gestational week in Fig. 3. The proportion of AFP-L2 was 98% in the 6th gestational week and disappeared around the 18th gestational week. The proportion of AFP-L3 reached a maximum of 45% in the 7th～11th gestational weeks and then decreased with advancing gestation. The proportions of AFP-P1, AFP-P3f, AFP-P4 and AFP-P5 are plotted against the gestational week in Fig. 4. AFP-P1 was present only in earlier samples obtained in the 6th～7th gestational weeks (0.7～4.6%), AFP-P3f was observed from the 6th(22.5%) to 11th gestational weeks (7.6%). The proportion of AFP-P4 decreased from 32% to 9% with

increasing gestational week, with a peak observed around the 7th~11th gestational weeks. The proportion of AFP-P5 was as high as 60% in the 6th gestational week and gradually decreased to 5% at near term. The proportions of AFP-A1 and AFP-A1s are plotted against the gestational week in Fig. 5. AFP-A1 was observed until the 16th gestational week (0.9~8.3%). AFP-A1s was present in samples of the 7th (1.9%), 15th (3.3%) and 16th (4.2%) gestational weeks.

In a yolk sac tumor, the proportion of AFP-C1 was 51%, AFP-L2 31%, AFP-L3 69%, AFP-P1 20%, AFP-P3f 8%, AFP-P4 37%, AFP-P5 35%, AFP-A1 7% and AFP-A1s 3%. The patterns of AFP bands in yolk sac tumor resembled those of amniotic fluids at the 6~7th gestational weeks.

Discussion

The sugar chain heterogeneity of human AFP has been analyzed mainly by column affinity chromatography (3), tandem crossed immunoelectrophoresis (4) and crossed-line affinity immunoelectrophoresis (5-9). These methods had insufficient sensitivity to analyze low levels of AFP as might be found in early gestational stages. Our technique of affinity electrophoresis coupled with antibody-affinity blotting allowed us not only to increase the sensitivity in detecting separated AFP bands but also to make a direct comparison of band mobilities by parallel running of samples (12).

Brębborowicz and Mackiewicz (4) showed the presence of Con A-nonreactive fraction and LCA weakly-reactive fraction in sera from patients with yolk sac tumors and in amniotic fluids. Following that study, Kelleher et al. (3), Mackiewicz et al. (4), Ishiguro et al. (5,6),

Nørgaard-Pedersen et al. (7) and Toftager-Larsen et al. (8-10) studied with lectins such as Con A, LCA and E-PHA. In addition to these lectins, we used allo A.

Reaction of lectins with glycoproteins is due to the carbohydrate moiety of AFP; hence the observed heterogeneity indicates different glycosylation of AFP depending on the stage of fetal development. In amniotic fluid, the percentages of Con A-nonreactive AFP-C1 was 85% in the 6th gestational week and then decreased to 15% in the 16th~17th gestational weeks and was hardly recognized at near term after 36th week and the percentages of AFP-L2 reaches 98% in the 6th gestational week. In early gestational weeks, the glycoform of AFP in amniotic fluid resembles that of AFP in fetal intestine and fetal kidney rather than that of AFP in fetal liver. Because Mackiewicz et al. (4) reported the percentages of Con A-nonreactive AFP, which they expressed as AFP-N-C and corresponds to AFP-C1 in our study, as 50% in amniotic fluid in the 6th gestational week and 10% in fetal liver, 80% in fetal intestine and 100% in fetal kidney in the 8th gestational week and that of LCA strongly-reactive AFP, which they expressed as AFP-RS-L and corresponds to AFP-L3 in our study, as 25% in amniotic fluid in the 6th gestational week. Further they reported the percentages of LCA weakly-reactive AFP, which they expressed as AFP-RW-L and corresponds to AFP-L2 in our study, as 50% in amniotic fluid in the 6th gestational week, 0% in fetal liver, 90% in fetal intestine and 100% in fetal kidney in the 8th gestational week. The percentages of AFP-C1 in amniotic fluids of early gestational stages in our study were 64~85% and were slightly higher than those of their study within a range of experimental error or variation. Moreover, the percentages of AFP-C1 in amniotic fluids are always

higher than the percentages of AFP-C1 in AFP produced by fetal livers throughout the first trimester and the second trimester (15). If the AFP present in amniotic fluid is a simple mixture of AFP glycoforms produced by the various fetal organs, such as liver, intestine and kidney through fetal urine and since the glycoform of AFP at near term is the maturest glycoform, it would indicate that the fetal liver develops prior to other fetal organs.

Since the presence of the bisecting *N* -acetylglucosamine (GlcNAc) at the core β-mannose of the biantennary structures reduces the affinity of glycoproteins for Con A (AFP-C2) (16), AFP-C1 is considered to have a bisecting GlcNAc at the core β-mannose of the biantennary complex-type oligosaccharides or tri- and tetra- antennary structures (17). The LCA strongly-reactive fraction corresponding to AFP-L3 was showen by Aoyagi et al. (18) to have sugar chains fucosylated at the core of asparagine-linked GlcNAc without the bisecting GlcNAc as expected from the carbohydrate specificity of LCA (11,19). LCA weakly-reactive AFP-L2 possesses the bisecting GlcNAc in addition to the fucosylated core of asparagine-linked GlcNAc as revealed by its Con A-nonreactive nature (20,21). From the above findings, in amniotic fluids in early gestational stages, AFP which has triantennary or tetraantennary structures amounts to the major part and it decreases with advancing gestation. On the other hand, AFP which has biantennary structures increases with advancing gestation and amounts to the major part at near term. Total percentage of AFP-L2 and AFP-L3, which correspond to the fucosylated AFP at the GlcNAc of the core of asparagine-linked oligosaccharides, in the 6th gestational week decreases to a half around the 16th gestational week and further decreases gradually in the following gestational weeks in amniotic fluid.

Therefore, regardless of having structure of bisecting GlcNAc or not, most of AFP in early gestational weeks have fucose at the asparagine-linked core GlcNAc. Considering our result, it is reasonable to assume that AFP-C1 corresponds to AFP-L2 in amniotic fluid.

In amniotic fluid, the percentage of E-PHA strongly-reactive AFP-P5 was 60% in the 6th gestational week. The presence of this glycoform in more than 50% in amniotic fluids of the early stages of gestation characterizes the sugar chain of amniotic fluid in contrast to that of fetal liver AFP. In fetal liver, AFP-P5 is present in as much as 10% during the whole studied gestational weeks without showing apparent peaks (15). The peak of AFP-P5 in fetal liver might have present before studied gestational weeks. The presence of α 2→3 sialylated outer galactose residue of the branch of mannose linked α 1→6 to the β-linked mannose as well as the bisected biantennary structure of the sugar chain constitutes the oligosaccharides of AFP-P5. It is interesting to know whether the AFP-P5 is due to the presence of α 2→3 sialylated galactose of the mannose linked α 1→6 arm or to the presence of bisecting GlcNAc. It is more reasonable to assume that AFP-P5 in amniotic fluids of early geatation has bisected structures in view of the high percentages of AFP-C1 and AFP-L2. In comparison of percentages of AFP-P5 and AFP-L2, it is hard to explain why AFP-L2 is present twice the percentage of AFP-P5 in the 6th gestational week. The percentages of E-PHA less strongly-reactive AFP-P4 were 12% in the 6th gestational week and once increased to 32% with a peak around the 7th~11th gestational weeks and decreased with increasing gestational week. The presence of the asialo AFP or asialylated galactose of the mannose linked α 1→6 arm, neither of the structures has the bisecting GlcNAc, gives rise to

AFP-P4 (22). Considering the percentages of AFP-C1 and AFP-P5, the glycoform which has both biantennary structure and the structure of α 2→3 sialylated galactose of the mannose linked α 1→6 arm amounts to at least 52% of AFP-C1 in the 6th gestational week. In amniotic fluid, the changes of the percentages of AFP-P4 are similar to those in fetal liver (17) throughtout pregnancy.

In early gestational weeks, E-PHA-nonreactive AFP-P1 and weakly-reactive AFP-P3f were recognized in the amniotic fluid but not recognized in fetal liver (15).

Only in the limited period of early gestation, allo A weakly-reactive AFP-A1s, which corresponds to the structure of asialo AFP, appeared and the percentages of AFP-A1s were less than 10%.

The glycoforms of serum AFP from a patient with yolk sac tumor in this study showed increased proportions of AFP-C1 and AFP-P5 as well as appearances of AFP-L2, AFP-P1, AFP-P3f and AFP-A1s, confirming the results reported previously (14,23) and these changes corresponded well to those of AFP from amniotic fluids of early gestational weeks.

We have shown in this paper that because the sugar chain heterogeneity of human AFP was obserbed in the AFP from amniotic fluids throughout pregnancy especially in the early gestational weeks, lectin-affinity electrophoretic analysis of amniotic fluid AFP was useful for the evaluation of developmental state of fetus with respect to AFP sugar chain.

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Fig. 1 Patterns of AFP bands separated by affinity electrophoresis with four lectins and detected by antibody-affinity blotting for amniotic fluids of different gestational weeks (Gest.W.) and serum from a patient with yolk sac tumor (Y. T.).

Fig. 2 Percentages of AFP-C1 band in amniotic fluids.

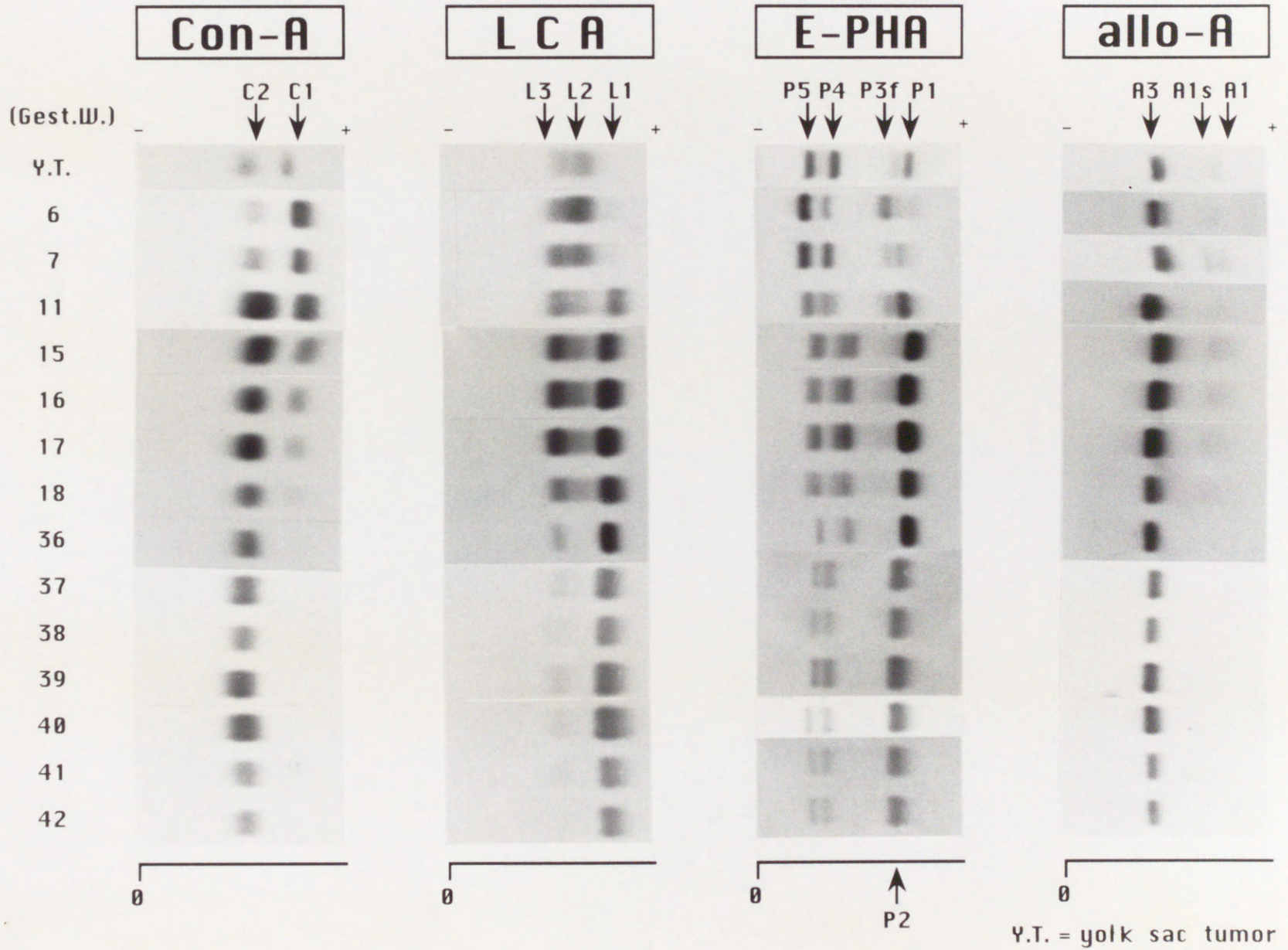
Fig. 3 Percentages of AFP-L2 and L3 bands in amniotic fluids.
○: AFP-L2 and ●: AFP-L3.

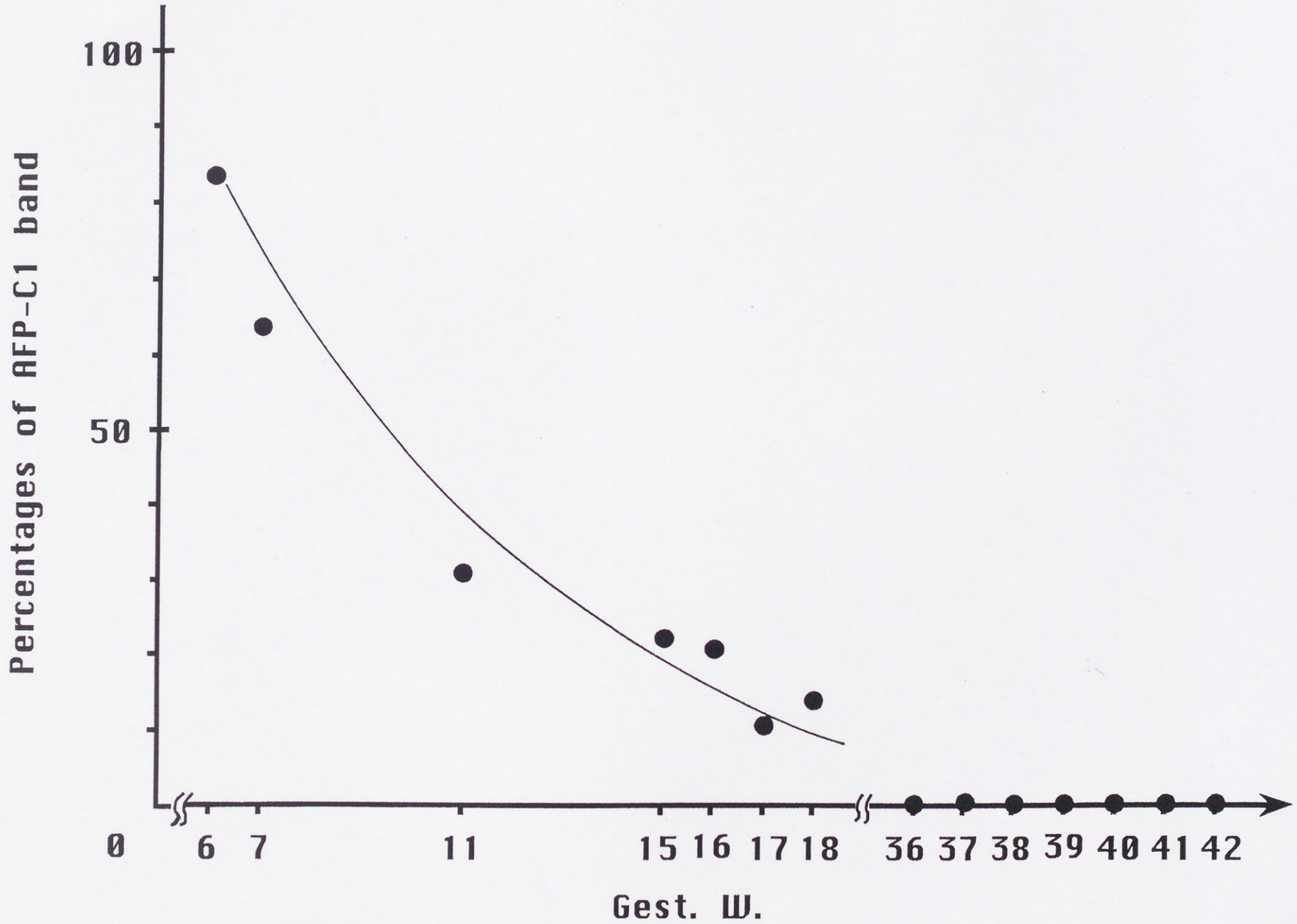
Fig. 4 Percentages of AFP-P1, P3f, P4 and P5 bands in amniotic fluids.
□: AFP-P1, ■: AFP-P3f, ○: AFP-P4 and ●: AFP-P5.

Fig. 5 Percentages of AFP-A1 and A1s bands in amniotic fluids.
○: AFP-A1 and ●: AFP-A1s.

Fig. 6 Proposd carbohydrate chain structures of AFP bands separated by four rectins.

GlcNAc = *N*-acetylglucosamine
Fuc = Fucose
N = Neu5Acα2 = Sialic acid
Man = Mannose
Asn = Asparagine
Gal = Galactose


Con-A
L C A
E-PHA
allo-A
(Gest.W.)
C2 C1
L3 L2 L1
P5 P4 P3f P1
A3 A1s A1
Y.T.
6
7
11
15
16
17
18
36
37
38
39
40
41
42
0
P2
Y.T. = yolk sac tumor


Percentages of AFP-C1 band
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50
0
6
7
11
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16
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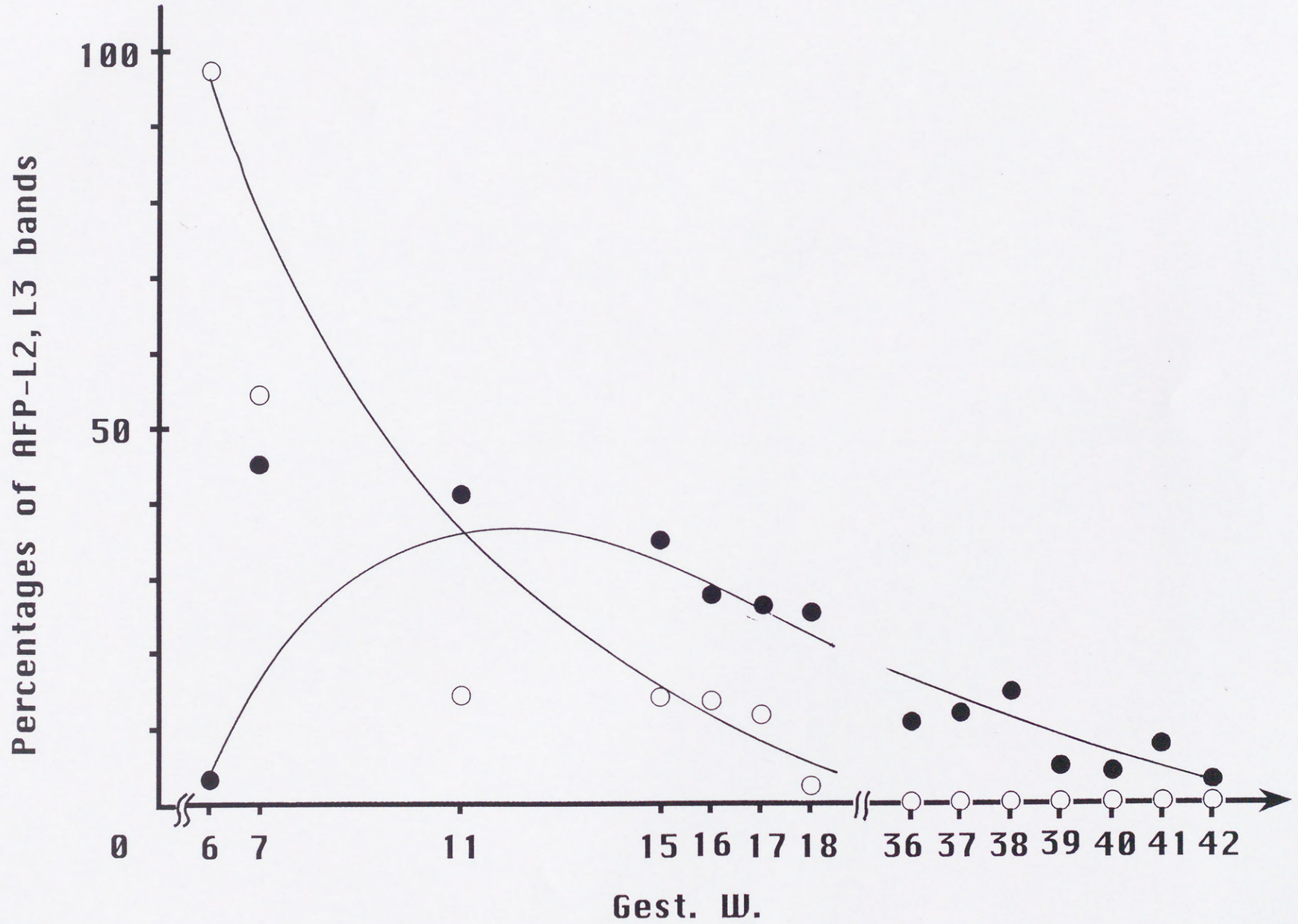
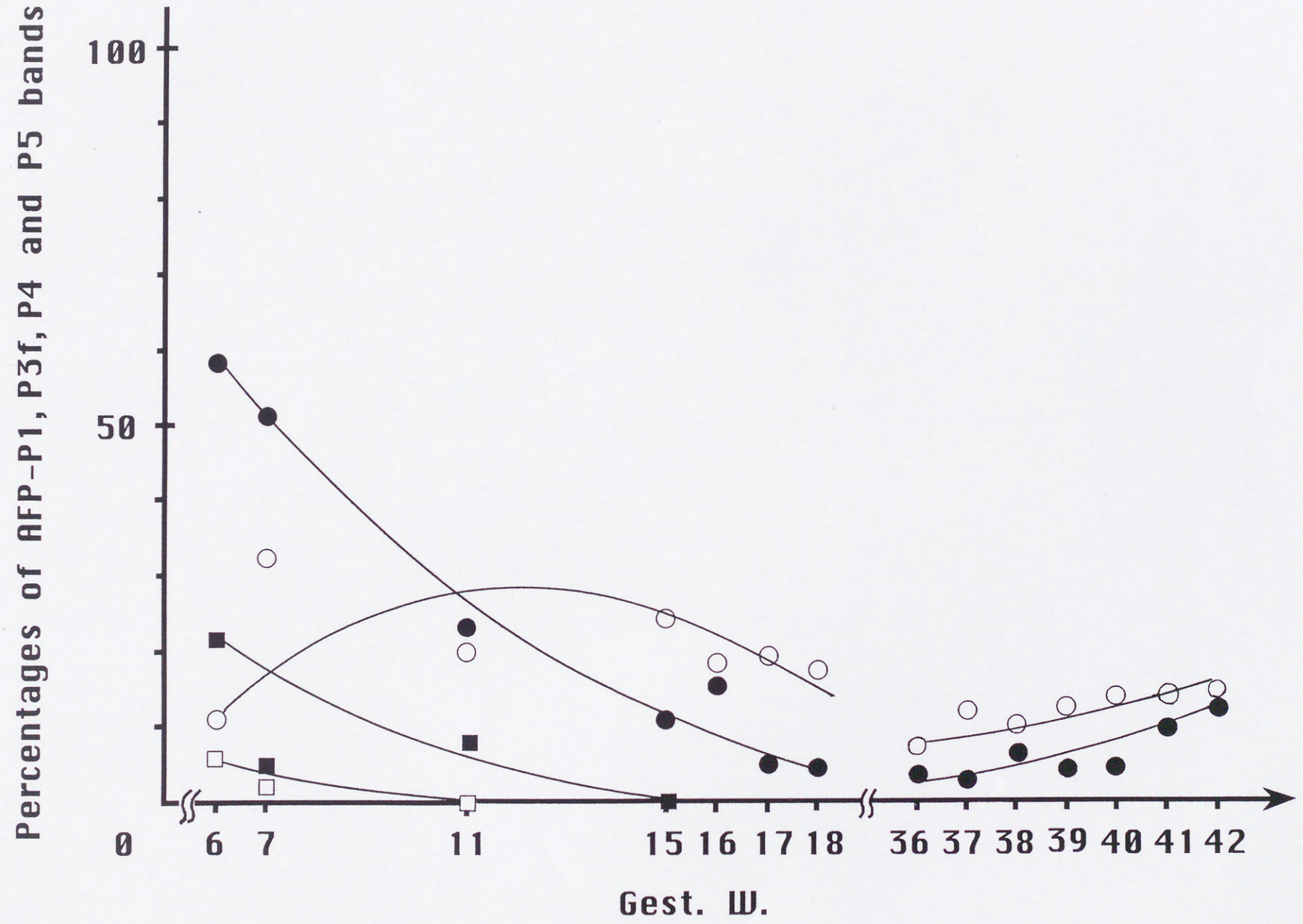
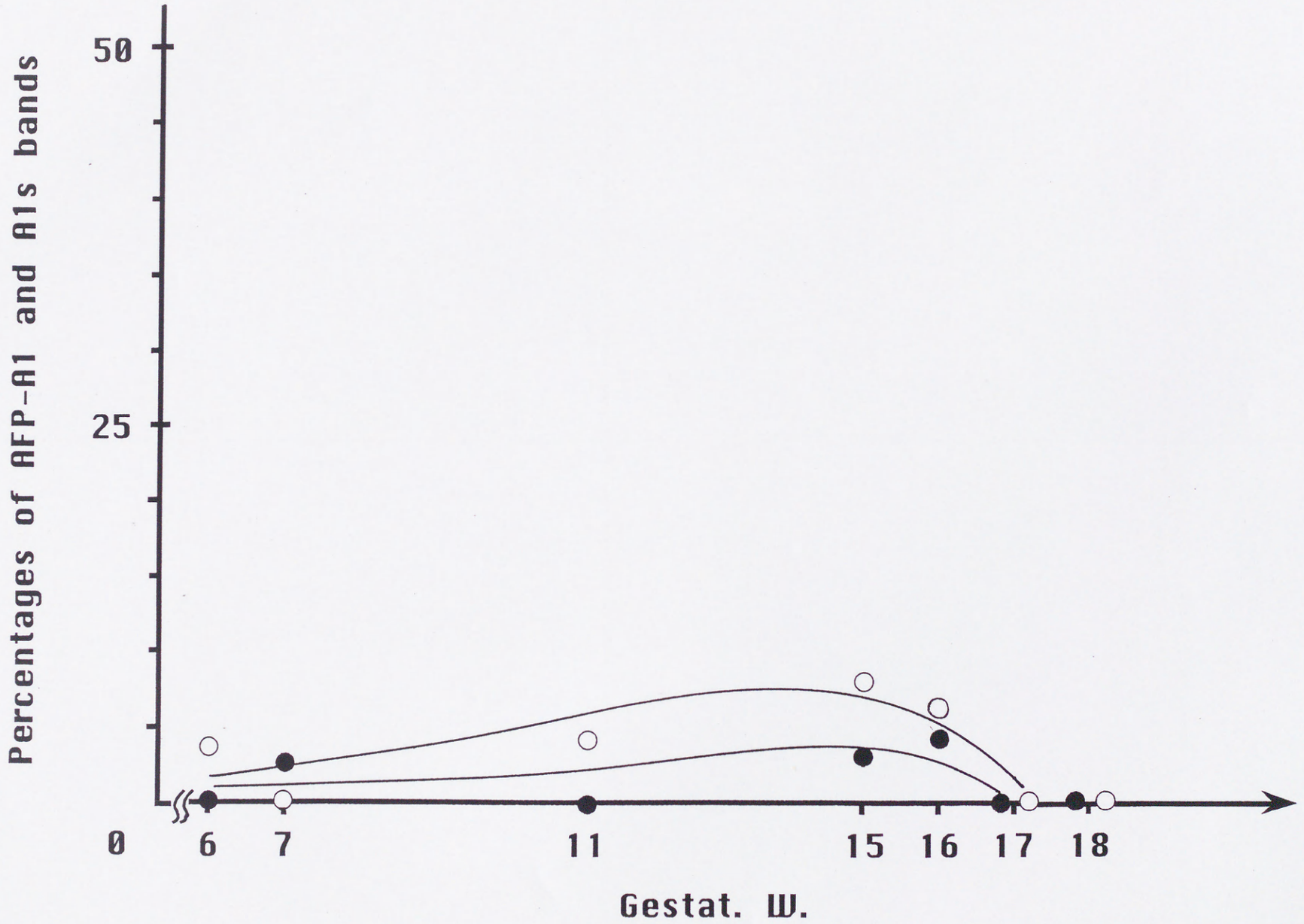
36
37
38
39
40
41
42
Gest. W.


100
50
0
Percentages of AFP-L2, L3 bands
6
7
11
15
16
17
18
36
37
38
39
40
41
42
Gest. W.


Percentages of AFP-P1, P3f, P4 and P5 bands
100
50
0
6 7 11 15 16 17 18 36 37 38 39 40 41 42
Gest. W.


Percentages of AFP-A1 and A1s bands
50
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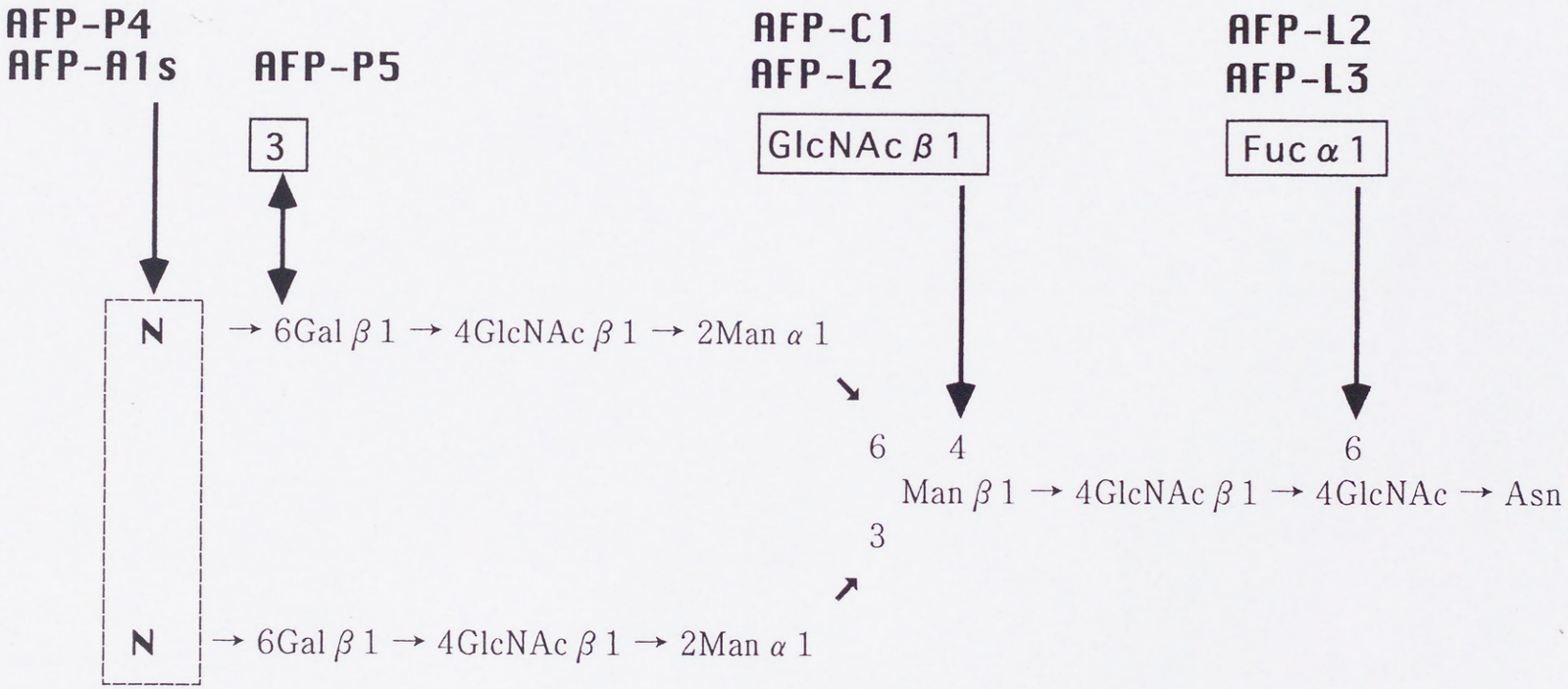
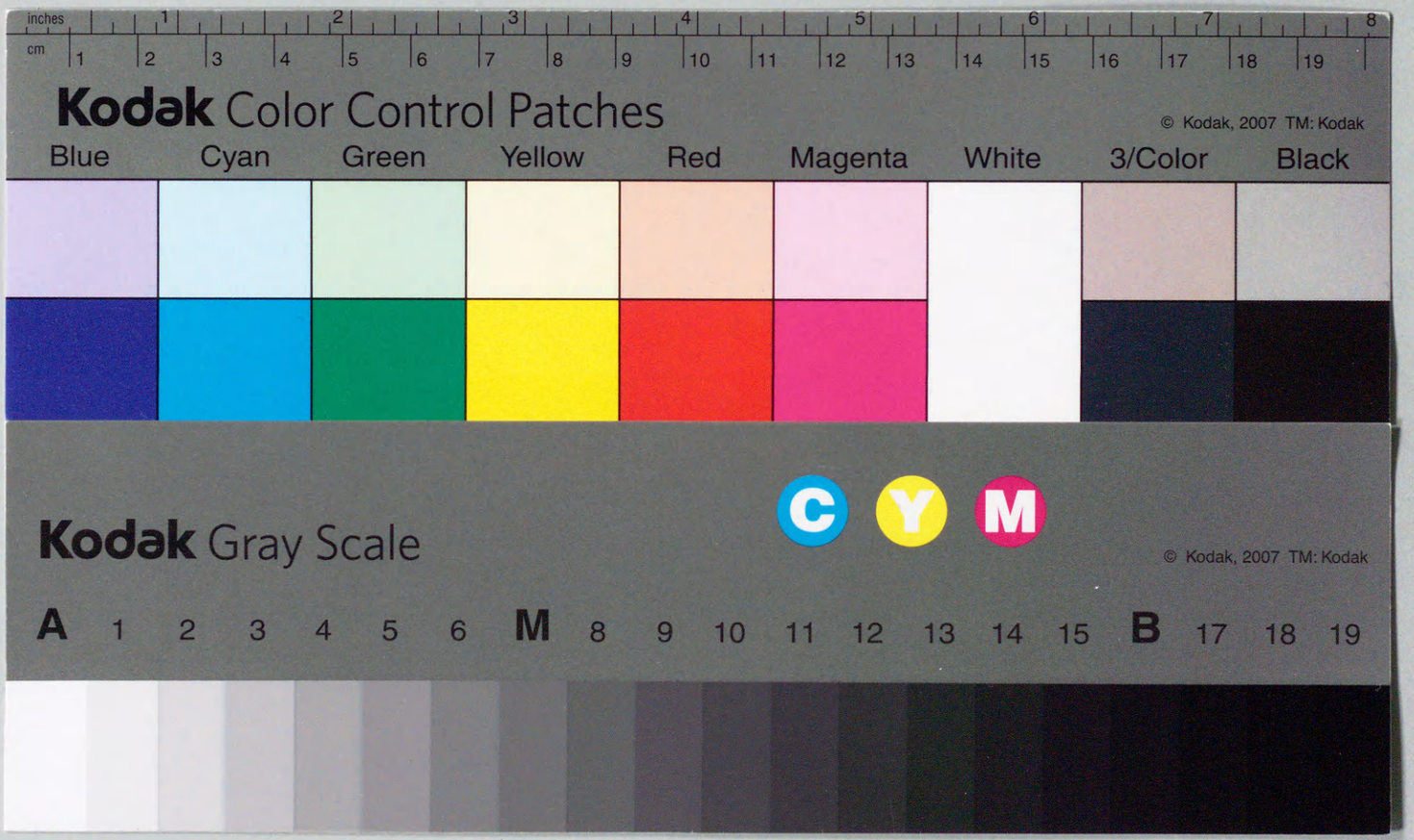
0
6
7
11
15
16
17
18
Gestat. W.


AFP-P4
AFP-A1s
AFP-P5
AFP-C1
AFP-L2
AFP-L2
AFP-L3
3
GlcNAc β 1
Fuc α 1
N
→ 6Gal β 1 → 4GlcNAc β 1 → 2Man α 1
6
4
6
Man β 1 → 4GlcNAc β 1 → 4GlcNAc → Asn
3
N
→ 6Gal β 1 → 4GlcNAc β 1 → 2Man α 1


inches
cm
Kodak Color Control Patches
© Kodak, 2007 TM: Kodak
Blue
Cyan
Green
Yellow
Red
Magenta
White
3/Color
Black
Kodak Gray Scale
C
Y
M
© Kodak, 2007 TM: Kodak
A 1 2 3 4 5 6 M 8 9 10 11 12 13 14 15 B 17 18 19